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DEVELOPMENT OF A MICROFLUIDIC APPROACH FOR THE REAL-TIME ANALYSIS OF AUTOCRINE AND PARACRINE TGF-β SIGNALING

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Sommario

Il Trasforming Growth factor β (TGF-β) è una citochina multifunzionale che controlla differenti processi cellulari tra cui proliferazione, differenziamento, migrazione e apoptosi. Sebbene il TGF-β controlli una varietà di processi biologici, la via di segnale è espressa in una maniera relativamente semplice. Il TGF-β è secreto come complesso latente e richiede un'attivazione enzimatica per legare il suo recettore e mediare il segnale intracellulare. Il controllo temporale della via di segnale è essenziale per regolare il processo di embriogenesi e il mantenimento dell'omeostasi nel tessuto maturo. Disregolazioni della via di segnale sono associate a condizioni patologiche come fibrosi e carcinogenesi.

La prima parte di questo lavoro è focalizzata sulla via di segnale di TGF-β. Esponendo cellule integrate in una piattaforma microfluidica ad amministrazioni multiple e periodiche di TGF-β, è stato identificato come differenti stimolazioni con TGF-β sono associate a differenti dinamiche della via di segnale ed espressione di geni target. In particolare è stata focalizzata l'attenzione sulla proteina SMAD7, responsabile dell'inattivazione della via di segnale ed è stato dimostrato che l'espressione di SMAD7 è indipendente dalla frequenza di stimolazione con TGF-β.

Differenti risultati clinicamente rilevanti possono essere ottenuti controllando l'attivazione, durata e intensità della via di segnale a livello intracellulare ed extracellulare, quindi, utilizzare come bersaglio la via di segnale di TGF-β nel trattamento farmacologico di patologie epatiche costituisce un approccio innovativo.

Nella seconda parte di questo lavoro, grazie all'utilizzo della tecnologia microfluidica è stato prodotto un dispositivo per analizzare alcuni aspetti del processo di fibrosi epatica *in vitro*. In questa piattaforma microfluidica il microambiente promuove l'accumulo di fattori profibrotici o antifibrotici in risposta a stimoli profibrotici o antifibrotici, secreti da differenti tipi cellulari, coinvolti nel processo di fibrosi epatica. È stata valutata la produzione , attivazione e funzione di TGF-β endogeno da cellule del microambiente epatico, con particolare attenzione su cellule stellate e macrofagi, che sono rispettivamente responsabili del processo fibrotico e infiammatorio. È stato inoltre analizzato lo specifico effetto della glicoproteina di matrice Trombospondina-1 nell'attivazione del TGF-β in forma latente. La Trombospondina-1 è incrementata nel siero di pazienti fibrotici, per questa ragione è stato valutato l'effetto della trombospondina-1 nell'attivazione del TGF-β autocrino secreto dalle cellule stellate epatiche. È stata inoltre analizzata la produzione di collagene mediata da TGF-β in cellule epatiche e cellule stellate, rilevando come queste ultime producano collagene in risposta a TGF-β endogeno o esogeno.

La terza parte del lavoro si è focalizzata sullo sviluppo di un modello di epatocita umano derivato da cellule staminali umane pluripotenti indotte utilizzando piattaforme microfluidiche. Il protocollo di differenziamento è stato utilizzato per ottenere cellule epatiche funzionali e ottimizzato per l'ottenimento di cellule epatiche in cultura su matrici acellulari derivate dal processo di decellularizzazione di fegato. È risaputo che il TGF-β induce un effetto di rimodellamento della matrice extracellulare e la deposizione di collagene, tuttavia non è noto l'effetto che la matrice extracellulare induce sulle cellule durante il processo fibrotico. In tale contesto matrici decellularizzate di fegato umano sono

state ottenute da donatore sano o paziente fibrotico per analizzare l'effetto della differente composizione della matrice extracellulare sulla secrezione di fattori endogeni mediata da cellule epatiche, rilevando un effetto sulla modulazione della produzione di TGF-β.

Summary

Transforming Growth factor β (TGF-β) is a multifunctional cytokine that controls several cellular processes including cell proliferation, differentiation, migration and apoptosis. Despite TGF-β mediates a variety of biological processes, the signaling is spread in a relatively simple manner. TGF-β is secreted as a latent complex and requires an enzymatic activation to bind its receptor and promote intracellular signaling. Temporal control of TGF-β signaling is essential to regulate embryogenesis and also to maintain homeostasis in mature tissue. Pathway dysregulation is associated to pathological conditions, such as fibrosis and carcinogenesis.

The first part of this work is focalized on TGF-β signaling pathway dynamics. By exposing cells integrated in a microfluidic platform to multiple, periodic administrations of TGF-β, it was identified how different TGF-β stimulations are associated to different pathway dynamics and expressions of target genes. In particular the analysis is focused on SMAD7 protein, responsible of pathway inactivation and it was demonstrated that SMAD7 expression is indipendent from the frequency of TGF-β stimulation.

Different clinically relevant outcomes can be obtained by controlling TGF-β signaling pathway activation, duration and amplitude via intracellular and extracellular mechanisms, thus, targeting the TGF-β signaling in the pharmacological treatment of liver diseases constitute a novel approach. In the second part of this work, taking advantage of the microfluidic technology, a novel device to recapitulate *in vitro* the main features of hepatic fibrosis was created. In

this microfluidic platform, the confined microenvironment promotes the accumulation of profibrotic or antifibrotic factors in response to profibrotic or antifibrotic stimuli, secreted by different kinds of cells involved in hepatic fibrosis. Moreover, the production, activation and function of endogenous TGF-β from cells in the hepatic microenvironment was analysed, with specific focus on hepatic stellate cells and macrophages that are respectively responsible of the fibrotic and inflammatory process. It was also analysed the effect of Thrombospondin-1, a specific extracellular matrix glycoprotein, in the activation of the TGF-β latent complex. Thrombospondin-1 is found to be increased in the serum of fibrotic patients and for this reason, the effect of thrombospondin-1 in the activation of autocrine TGF-β secreted from hepatic stellate cells was evaluated. Finally it was analysed the collagen production mediated by TGF-β in hepatic cells and stellate cells, revealing that the latter produce collagen in response to endogenous or exogenous TGF-β.

The third part of the work involved the development of a human inducible Pluripotent Stem Cells (iPSCs) derived hepatocyte model, using microfluidic platforms. The differentiation protocol was applied to obtain functional hepatic-like cells and also optimized for the obtainment of hepatic cells cultured on acellular matrix derived from liver decellularization process. It is well-known that TGF-β exert an effect of extracellular matrix (ECM) remodelling and collagen deposition, nevertheless is not known the effect that ECM induces on cells during fibrotic process. In this context decellularized extracellular matrix was also derived from healthy donor or fibrotic patients in order to analyse the effect of ECM composition on secretion of endogenous factors mediated by hepatic cell, revealing an effect on modulating TGF-β production.

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State of the Art and motivation

Transforming growth factor β (TGF-β) belongs to a class of pleiotropic cytokines that are involved in the process of embryonic development, wound healing, cell proliferation and differentiation. Moreover TGF-β is a central regulator in the pathogenesis of liver disease, because it contributes to almost all of the stages of disease progression. Although it has been extensively demonstrated that the TGF-β signaling pathway upregulation exerts abundant profibrotic effects, the mechanism of the signaling transduction is still poor understood and need to be more investigated to support the development of new antifibrotic therapeutic strategy.

1.1 Plasticity of TGF-β signaling

The Transforming Growth factor β (TGF-β) pathway controls several cellular processes, including cell proliferation, differentiation, migration and apoptosis, during embryogenesis as well as in mature tissue. The discovery of TGF-β dates back to 1978, with the detection by De Larco and Todaro of a secreted factor produced by virally trans-formed fibroblasts. This factor, released in the culture

media, act as a “non-cell autonomous oncogene” inducing normal fibroblast to grow in soft agar. The TGF-β superfamily of secreted factors is comprised of over 30 members including Activins, Nodals, Bone Morphogenetic Proteins (BMPs), and Growth and Differentiation Factors (GDFs). An important aspect in TGF-β research focuses on the role of this signaling cascade in embryonic stem (ES) cells. In mouse Embrionic Stem Cells (ESCs) TGF- β promotes differentiation, in contrast, in human ESCs promote the maintenance of the pluripotency, while BMPs prompt their differentiation. Positive and negative regulations of TGF-β ligands can redirect pluripotent cells toward progressively more committed fates; indeed TGF-β/BMP agonists or antagonists are elements of the cocktails of extrinsic factors currently employed in the manipulation of ESCs and Induced Pluripotent Stem Cells (iPSCs) for future regenerative medicine applications [1]. Temporal control of TGF-β signaling is essential to regulate embryogenesis and also to maintain homeostasis in mature tissue. Pathway dysregulation is associated to pathological condition, like fibrosis and carcinogenesis.

1. 2 Mechanism of TGF-β signaling

The TGF-β cytokines family comprises six conserved cysteine residues, are encoded by 42 open reading frames in human, 9 in fly, and 6 in worm [2]. It contains two subfamilies, the TGF-β/Activin/Nodal subfamily and the BMP (bone morphogenetic protein)/GDF (growth and differentiation factor)/MIS (Muellerian inhibiting substance) subfamily, as defined by sequence similarity and the specific

signaling pathways that they activate. Although the diverse TGF-β ligands elicit quite different cellular responses, they all share a set of common sequence. The active form of a TGF-β cytokine is a dimer stabilized by hydrophobic interactions, which are further strengthened by an inter subunit disulphide bridge in most cases. The dimeric arrangement of the ligands suggest the formation of a complex with two type I and two type II receptor. [3]. The activation of the type I receptor involves the phosphorylation of its GS domain by the type II receptor. Beyond the core components of this signaling pathway many other factors modulate the signal and thereby contribute to the versatility of the response. At the membrane level, the access to receptor is controlled by soluble proteins that sequester TGF-β ligand (i.e. decorin) [4-5], and by membrane-bound co-receptors that promote binding (i.e. betaglycan) [6-7]. The receptor activity is further regulated by several receptor internalization routes [8], and by receptor turnover. Binding to the extracellular domains of both types of the receptors by the dimeric ligand induces a productive conformation for the intracellular kinase domains of the receptors, inducing the phosphorylation and subsequent activation of the type I receptor. The type II receptor kinases are thought to be constitutively active, although the regulation of this process remains unclear.

1.3. Canonical and non-canonical TGF-β signaling pathway

The canonical TGF-β signaling pathway requires ligand binding to a heteromeric complex of type 1 and 2 serine/threonine kinase receptors. TGF-β ligand initiates the signaling cascade by binding to and bringing together type I and type II receptor serine/threonine kinases on the cell surface. This allows receptor II to phosphorylate the receptor I kinase domain, which then propagates the signal.

The primary intracellular mediators of TGF-β signaling are the Smad proteins, which are classified in receptor-regulated Smads (R-Smads), the Co-mediator Smad (Co-Smad) and the inhibitory Smad (I-Smad). R-Smads (1,2,3,5 and 8) are directly phosphorylated by the type I receptor kinases and undergo homotrimerization with Smad 4. The activated complex translocates into the nucleus and regulates the transcription of target genes [9]. At the intracellular level, many processes require auxiliary proteins, such as SARA for the binding of R-Smad to the receptor and Schnurri for the binding of the R-Smad/Co-Smad complex to the DNA binding element [10]. The restriction of those auxiliary factors to specific cell-types will make the response cell context-dependent [11]. TGF-β can also activates non-SMAD signaling pathways, such as TAK1 (TGF-β-associated kinase 1), Erk (extracellular signal-regulated kinase), p38, MAPK (mitogen-activated protein kinase) and AKT [12-15] . Akt further signals via the mammalian target of rapamycin (mTOR) pathway to control cellular responses crucial for survival, growth, migration and invasion [16-17] .Moreover, TGF-β signaling output is strongly influenced by intersecting other signaling pathways, including Ras, Notch and Wnt, and by crosstalk between ligands, receptors and

Smads of the TGF-β superfamily, such as activins, BMPs and myostatin. Regarding canonical pathway, under continuous ligand stimulation, R-Smads remains phosphorylate with nuclear localization. Cells respond to the absolute number of bioavailable TGF-β molecules in their environment; sustained TGF-β stimulation result in a persistent Smad 2/3 phosphorylation, and also short pulse exposure of ligand can produce a sustained long-term signaling response. Short term TGF-β stimulation causes only transient Smad 2/3 phosphorylation and can be terminated by ligand depletion. Smad2 phosphorylation induced by TGF-β is graded in the short-term and ultrasensitive (switch like) in the long-term. The cell growth arrest in response to TGF-β shows switch-like rather than graded behaviour. Most of cell fate decisions regulated by TGF-β-related molecules are likely to be switch-like and irreversible (e.g. differentiation and apoptosis). Another aspect of TGF-β biology is how cells convert continuous ligand concentration into discontinuous cell fate decision. In the TGF-β signaling cascade, there is little evidence indicating the existence of strong positive feedback loops. Changes in ligand concentration result in faithful changes on the amount of nuclear Smad2 [18-19]. In agreement with the notion that there is no signal amplification in the TGF-β signaling cascade, short-term Smad2 phosphorylation in response to changes in TGF-β concentration in the culture medium is graded. Smad 2/3 activation reflects the level of ligand to which cells are exposed and will rise and fall with the levels of receptor activation. Despite Smad 2/3 exhibits stable nuclear accumulation under continuous TGF-β1 stimulation, it has been demonstrated that transcriptional response is transient and coincides with Smad 4 nuclear accumulation. Smad 4 kinetics is not dependent on ligand dose and continuous signaling from receptor [20]. Transient dynamic of

TGF-β pathway is correlated to transcriptional expression of Smad 7,that is quickly induced upon TGF-β stimulation. As a key negative regulator of TGF-β signaling, Smad 7 exerts its inhibitory effects by blocking receptor activity, inducing receptor degradation, interfering with Smad-DNA binding or interacting with receptor-regulated Smad [21-22].

1.4 Mechanism of TGF-β latent form activation

Although TGF-β synthesis and expression of its receptors are widespread, activation is localized to the sites where TGF-β is released from latency. Folding and secretion of active TGF-β1 requires the co-expression of their prodomains, whereas the TGF-β1 prodomains can be biosynthesized in the absence of the growth-factor domain. TGF-β gene encodes for a protein of 50 kDa (25 kDa dimer) that dimerizes with the proprotein cleaved by the endo-peptidase furin, but the dimeric proprotein remains non-covalently attached to the TGF-β 25 kDa dimeric protein. The covalently attached proprotein blocks TGF-β binding to its receptors , making the TGF-β latent. The dimeric pro-protein is called " latency-associated peptide" (LAP) (75 kDa) that once binding TGF-β is called "small latent TGF-β complex (SLC). This complex remains in the cell until it is bound by a third protein product called the latent TGF-β binding protein (LTBP). The SLC is made of a dimerized single gene product of the TGF-β pro-protein, non-covalently bound to active TGF-β. SLC is associated with a disulphide bond with LTBP forming the "large latent complex"(LLC). It is the LLC that is secreted

from cells that needs to be processed further to release active TGF-β [23] and the complex could be bound to collagen and other matrix proteins through binding sites on the LTBP [24]. Two different groups of TGF-β receptors are involved in the signaling activation. The first type recognizes and bind LAP as a part of the LLC or the SLC domain, while the second group binds active TGF-β and includes three type of receptors. The type III receptors are β-glycan and endoglin that could be in a soluble form or bound on the cell surface. In the first case they acts as an inhibitor of active TGF-β, preventing TGF-β from binding the type II receptor, while in the second case facilitates TGF-β binding to the Type II receptor by handing over the active TGF-β to the type II receptor. Once type II receptor bind active TGF-β, there is recruitment and activation of the type I receptors that initiates the Smads intercellular signaling pathway.

1.4.1 Enzyme-mediated and Receptor-mediated TGF-β activation.

Activation of TGF-β requires the release of TGF-β from the LAP and the LTBP in the LLC. This process would involve the release of the LLC from the matrix, to which it is attached, followed by a conformational change of further proteolysis of the LAP to release TGF-β to its receptors. The most common laboratory method of activating TGF-β involves a transient acid or heat treatment. These methods take advantage of the biophysical properties of TGF-β to resist proteolysis, temperature and extreme pH conditions. Treating cell culture supernatants with pH lower than 2.0 or a temperature of 95°C for a short period of time [25] activate

all the TGF-β in a sample and can be compared with the same sample not treated to quantify the level of activated and latent TGF-β in the sample. The physiological activation of TGF-β may involve surface and localized protease activity. Metalloproteinases release the LLC from the tissue matrix [26-29]. The proteases cut LLC in a hinge region in the LTBP between the matrix-binding domains and the remaining domains of the LTBP that include the LAP-binding domain. When freed from the matrix the LAP can bind surface receptors and undergo conformational changes that release TGF-β. An important enzyme involved in the activation of TGF-β is plasmin, that is converted from plasminogen at the cell surface by a receptor-bound urokinase plasminogen activator or part of platelet activation [30]. The plasmin system of TGF-β activation was suspected to be the pathway by which TGF-β stored in platelets is activated and released in clot formations. Activation by plasmin is a complex process requiring interactions of the latent complex with cells through binding of the mannose-6-phosphate residues of LAP to mannose-6-phospate receptors and the activity of tissue transglutaminase. Plasmin might only activate the large form of the latent complex, however, platelet activation of TGF-β may more involve furin-like endopeptidases [31].This pathway is the major reason for the high levels of TGF-β in serum, which can influence TGF-β sensitive culture conditions containing serum. The receptor-mediated TGF-β activation involve the bind of LAP on cell surface making it possible for cells to deliver active TGF-β in an autocrine or paracrine manner to its own or another cell's TGF-βRs. This process is seen with activated macrophages binding on its surface latent TGF-β from the environment or from its own production and activating TGF-β[32]. There are

several surface-binding proteins for LAP identified. They are thrombospondin-1 (TSP-1)/CD36, M6PR and multiple αV-containig integrins [33-36]. Binding of TGF-β to αvβ6 integrins through RGD sequence in the β1 LAP stimulate activation of TGF-β, potentially through conformational alterations of the integrin bound-latent complex exerted by cytoskeletal forces. Another potential mechanism for *in vivo* regulation of TGF-β involves reactive oxygen species [37]. It is proposed that site specific oxidation of certain amino acids in the LAP elicits a conformational change in the latent complex that releases free active TGF-β. Binding to the extracellular matrix protein thrombospondin-1 to the latent complex is also another mechanism of activation that induces a conformational rearrangement of the LAP in such a manner as to prevent the LAP from conferring latency on the mature domain of TGF-β.

1.4.2 Activation of Latent TGF-β by Thrombospondin-1

Thrombospondin (TSP) is a multifunctional protein that exists as both secreted and as an insoluble extracellular matrix molecule. Thrombospondin-1 (TSP-1) isoform is a major component of platelet α-granules that act as an immediate response gene being upregulated in response to serum and growth factors such as TGF-β and PDGF. The function of TSP within a particular context depends on the complement of TSP receptors that a particular cell express and the expression of other TSP binding molecules like fibronectin, plasminogen , type V collagen and TGF-β. Plateled TSP-1 causes growth inhibition of aortic endothelial cells at

least partially via a TGF-β sensitive mechanism. The TGF-β associated with thrombospondin is in an active form. Immunoprecipitation studies *in vitro* show that TSP and LAP can associate to each other and endothelial cell conditioned medium. Data from Bailly et al. [38] suggest that the LAP of the latent TGF-β complex can remain associated with TSP following activation of latent TGF-β. The persistence of TGF-β biological activity in complexes containing TSP and LAP suggest that this interaction may alter the conformation of the LAP in a way that it is unable to confer latency. The TSP-1 site responsible for activation consists of a three amino acid sequence (RFK) located between the first and the second type 1 repeats [39]. A second motif present in each of the three type 1 repeats, WxxW (WSPW, WSHW,WGPW) that binds to a site in the active domain of TGF-β and facilitate the ability of RFK to activate latent form, acting as a "docking site " to correctly orient the RFK sequence with its complementary site on the latent TGF-β molecule. Physiologic examples of TSP-regulated TGF-β activation involve wound healing process. Localized and increased TSP expression has a role in the wound healing process, as a result of platelet degranulation and growth factor stimulation [40]. Studies conducted in TSP1 null mice demonstrated that administration of TGF-β activating peptide from TSP1 restore the wild-type phenotype in dermal wounds in TSP1 null animals, with resolution of capillary angiogenesis and macrophage infiltration. Furthermore, addition of active TGF-β to the TSP-1 null mice restore the wild-type phenotype indicate that a major function of TSP-1 in wounds is to activate latent TGF-β. This activation system may be a useful therapeutic tool for increase TGF-β activity. Studies of the phenotypes of TGF-β null and TSP-1 null mice reveal similar histopathology in multiple organ system, in particular lung and pancreas.

This similarity in phenotype suggest that a major factor in the development of histologic abnormalities in TSP1 null mice is the lack of local active TGF-β in the absence of TSP-1. In the case of the bronchial epithelial alterations, both TSP-1 and TGF-β are expressed by epithelial cells, suggesting an autocrine pattern of regulation, while in the pancreas the islet cells express TSP-1 and the acinar cells express TGF-β1, indicating that TSP-1 can regulate latent TGF-β activation in a paracrine situation as well. TSP dependent activation of latent TGF-β is a process involved in some fibrotic disease like pulmonary fibrosis. In this pathology infiltrating macrophages secrete active TGF-β with temporal characteristics that correspond to increased expression of TSP1 protein by these cells. TSP-1 and CD36 interaction also activates the p38 MAPK pathway in kidney podocyte and is involved in podocyte apoptosis [41]. On macrophages TSP-1 binds to CD36 and leads to macrophage activation. In platelets TSP-1 activated CD36-dependent pathways including c-Jun N-terminal kinase and p38 MAPK and regulates platelets functions. Using anti-TSP antibody block the stimulation of TGF-β activity. Alveolar macrophage-derived latent TGF-β appears to be activated by a hybrid plasmin-TSP-dependent mechanism that involves latent TGF-β interaction with thrombospondin, bound to the cell surface molecule CD36 and cleavage by plasmin . Interaction of TSP secreted by activated macrophages with latent TGF-β are apparently not sufficient to activate latent TGF-β. Is it possible that other protein such as fibronectin, fibrinogen, or heparin/heparin sulfate that are also secreted by machrophages, may compete with TGF-β for the tryptophan-rich motifs that are apparently necessary for docking and orientation of TGF-β with thrombospondin.

1.5 TGF-β signaling in liver disease and fibrosis

TGF- β1 is one of the primary factor that drive fibrotic disease associated with the excessive deposition of extracellular matrix (ECM), resulting in an ongoing loss of normal tissue structure. About 60 other ECM-related genes were also identified as immediate-early genes targets downstream of TGF-β [42]. A wide range of animal studies have established TGF-β1 as the predominant regulator of fibrotic disease like tubule-interstitial fibrosis [43-47] making the TGF-β1 pathway downregulation an attractive target for pharmacological treatment. Despite these findings, TGF-β1 regulates many biological responses including cell proliferation, apoptosis, differentiation, autophagy and the immune response. TGF-β1 promotes fibrosis through both direct and indirect actions on various cell type, inducing cell proliferation, migration, activation and the transcription of profibrotic molecules including collagens, fibronectin and plasminogen activator inhibitor-1 (PAI-1) [48-50]. TGF-β1 can also induce a repair and/or fibrotic response through indirect mechanisms. For example it can induce a mesenchymal gene expression programme that induces the transition of epithelial, endothelial and fibroblast into α-smooth muscle actin (SMA)-expressing myofibroblasts [51]. Hepatic fibrosis is a reversible wound healing response to acute or chronic liver injury and is characterized by the accumulation of ECM [52]. The process following liver injury involves an acute and a chronic response [53]. When acute injury is not severe, neighboring adult hepatocytes are able to regenerate and replace apoptotic and necrotic cells. If the insult persists, the regenerative process fails and hepatocytes become substituted by extracellular matrix protein. During

chronic disease, the composition of the ECM changes from collagen type IV and VI, glycoproteins and proteoglycans into collagens type I and III and fibronectin. A key event in liver fibrosis includes the activation of HSCs, whereby these cells adopt a myofibroblast-like phenotype. Stimuli triggering HSC activation originating from injured hepatocyte, sinusoidal endothelial cells, kupffer cells and platelets lead to a morphological changes in HSC shape , loss of vitamin A and the expression of cell surface receptors for growth factors and cytokines. The perpetuation of hepatic stellate cell activation is governed by PDGF and other cytokine. Other mitogens that can modulate HSC proliferation via paracrine signaling are TGF-α and epidermal growth factor. TGF-β is a key molecule during the progression of chronic liver disease, as it is the most potent stimulus for the production of collagen I and other ECM components, including fibronectin and proteoglycans. The resolution of liver fibrosis and cirrhosis observed in animals and humans has been well studied. This process may be explained by the HSCs reversion into a quiescent stage and/or apoptosis. TGF-β signaling is upregulated and activated in fibrotic diseases and modulates fibroblast phenotype and function, inducing myofibroblast transdifferentiation while promoting matrix preservation. The profibrotic actions of TGF-β are mediated, at least in part, through the induction of its downstream effector like Connective Tissue Growth Factor. During the progression of various liver diseases, such as chronic virus hepatitis, alcoholic liver diseases, non-alcoholic steatohepatitis, hepatic fibrosis, and hepatocellular carcinoma, TGF-β is involved from the initial liver injury to end-stage [54]. For this reason, targeting TGF-β signaling is one of the major tools for the treatment of fibrotic disease. Inhibition of TGF-β1 could be achieved by a variety of approaches; however, progress in translating findings from basic

research into the clinic it was a very slow process. This lack of progress in large part relates to concerns that inhibition of TGF-β in regulatory T cells could exacerbate autoimmune disease. In this contest understanding how TGF-β signaling pathway works in different type of cells is essential to elaborate a functional therapy to eliminate the harmful effect mediated by the upregulation of the signaling without affecting the normal control of the process that regulate cellular homeostasis.

1.6 Thrombospondin- 1 in liver disease and fibrosis

Thrombospondin is mostly produced by platelets, however, it can also be produced by different types of cells, including hepatocytes, stellate cells, megakaryocyte, vascular smooth muscle cells, fibroblast, endothelial cells, epithelial cells and keratinocytes. In the healthy liver TSP-1 is expressed at a very low level but can be up-regulated under pathophysiological conditions. Hepatocytes from liver tissue of patients with congenital hepatic fibrosis showed stronger staining for TSP-1 than normal tissue. Stellate cells derived from fibrotic patients also secrete more TSP-1 [55]. TSP-1 expression is also increased in stellate cells derived from diet induced fibrosis mouse models and also in human patients with alcoholic cirrhosis. These evidence suggest that TSP-1 mediated TGF-β activation contributing to the development of liver fibrosis. This was further supported by a study showing that injection of the LSKL peptide to block TSP-mediated TGF-β activation reduced dimethyl-nitrosamine-induced liver

damage and fibrosis suggesting that blocking TSP-mediated TGF-β activation may serve as a novel therapeutic target for liver fibrosis.

1.7 Therapeutic approaches to the treatment of liver fibrosis

Nowadays there is no standard treatment for liver fibrosis. Although experimental studies have revealed targets to prevent fibrosis progression in rodents, the efficacy of most treatments has not be proven in humans [56]. The ideal antifibrotic therapy would be one that is liver-specific and effective in attenuating excessive collagen deposition without affecting normal ECM synthesis. The removal of causative agent is the most effective intervention in the treatment of liver fibrosis in chronic liver diseases, this strategy provide also liver transplantation for patients with cirrhosis and clinical complications. The main antifibrotic drugs in development, for the treatment of liver fibrosis involve several mechanism: decrease of oxidative stress, inhibition of inflammatory response and inhibition of HSCs activation and also function. Phosphatidylcholine and S-adenosyl-methionine are two example of antioxidant molecules involved in the reduction of oxidative stress. Because inflammation precedes and promotes the progression of liver fibrosis Colchicine and Corticosteroids has been proposed as anti-inflammatory drugs. Angiotensin inhibitors and TGF-β1 inhibitors are also involved in inhibition HSCs activation and function. The inhibition of the renin-angiotensin system is probably one of the most promising strategy in treating liver fibrosis because they are still widely used as anti-fibrotic agents in

patients with chronic renal and cardiac diseases [57]. Regarding TGF-β1, this is the primary factor that drives fibrosis in many organs, especially for chronic kidney disease [58]. TGF-β1/Smad signalling pathway is the major driver of renal fibrosis and is, therefore, a clear therapeutic target to prevent fibrosis in several organs like liver. Inhibition of TGF-β1 signalling could be achieved by a variety of approaches; however, progress in translating findings from basic research into the clinic has been disappointingly slow because inhibition of TGF-β signaling may lead to unknown adverse reactions.

1.8 TGF-β as a therapeutic target

The blockade of TGF-β and its signaling pathway provides multiple opportunities in drug design. The current strategies to alter the TGF-β signaling refer to three levels: ligand, receptor-ligand interaction and intracellular signal transduction. At ligand level, it is possible to target the transcriptional action of TGF-β and the maturation process of latent TGF-β by RNA interference using two type of molecules, that is micro interfering RNA (miRNA) and short interfering RNA (siRNA). Antisense oligonucleotides (ASOs) to RNAs and the targeting of TGF-β mRNA allow the silencing of these genes. Trabedersen (AP-12009), a synthetic 18-mer phoshorothioate ASO, has progressed to a Phase II clinical trial for oncology applications [59]. Intervention at the ligand-receptor level comprises three categories of compounds: monoclonal Antibodies (mAbs), natural TGF-β inhibitors, and soluble TGF-β receptors. The mAbs include CAT-152, CAT-192

and GC1008, which have been already tested for the treatment of fibrotic disorders and cancer. Soluble TGFβRIII has been demonstrated to exhibit efficacy in inhibiting the growth and angiogenesis of human colon and breast cancer. Intervention at the intracellular signaling level can be mediated by drugs that block the intracellular kinase activity of TGF-β receptors. Also other type of drugs can target the interaction of Smads with TGF-β receptors through the use of peptide aptamers to Smads. The currently used inhibitors consist of an imidazole scaffold, such as SB-431542 and SB-505124 or a pyrazole scaffold, such as LY-580276 and LY2109761. These drugs selectively blocks the activation of Smad2. This strategy aims to inhibit signaling activity by blocking the substrate-binding site of the TβRI kinase. Other strategies provide the modulation of various targets downstream of the receptor like vector encoding Smad 7 [60] or the C-terminal-truncated dominant-negative Smad 4 complementary DNA[61]. However, the overexpression of Smad 7 is difficult to take in consideration for clinical application because it facilitates the loss of TGF-β mediated growth inhibition due to the activation of Smad-indipendent TGF-β signaling pathway [62]. Although there was many clinical trial that involve TGF-β signaling pathway as a pharmacological target in liver disease, a drug design strategy involving the blockade of TGF-β signaling with low specificity could produce adverse reaction; the inhibition of TGF-β1 signaling in regulatory T cells could exacerbate autoimmune disease.. From this point of view it is essential to take into account the importance to continue the development of targeted drugs and understanding the complexity TGF-β signaling in different cell type and organism. In contrast to TGF-β1 deficiency, Smad3 deficiency is not lethal in mice, suggesting that Smad3 might be a better therapeutic target than TGF-β1[63]. The Smad3 inhibitor SIS3,

reduced renal fibrosis in a mouse model of diabetic nephropathy [64], also the expression of profibrotic miRNAs induced by Smad3 can be blocked by anti-miR therapy. On the other hand studies performed in animal models have demonstrated that overexpression of Smad7 can suppress renal fibrosis and reduce NFkB-driven inflammation [65-69]. On this point of view rebalance Smad3 and Smad7 in TGF-β pathway could be a novel approach to counteract the fibrotic process in a multitude of fibrotic disease.

1.9 In *vivo* models for liver fibrosis

Considerable efforts are currently involved to liver fibrosis research to elucidating the molecular mechanism that drive this disease and also in view of establishing effective diagnostic and therapeutic strategies. The investigation of the mechanisms leading to liver fibrosis is mainly based on the induction of fibrosis in animal models. Chemical-based models are commonly used to study particular pattern of lesions. Ethanol, carbon tetrachloride (CCl_4), thioacetamide, dimethylnitrosamine (DMN) and diethylnitrosamine (DEN) are the most used chemicals because of their reproducibility and appropriate reflect the mechanism involved in human liver fibrosis. The chemical-based animal models are popular because of their high reproducibility and appropriate reflect the mechanisms involved in human liver fibrosis [70].All this molecules injection are associated with several deleterious non physiological events such as the production of ROS, glutathione depletion, lipid peroxidation and increased collagen synthesis [71-72]. Hepatocytes are the main source of lipid peroxides and apoptotic bodies in injured

liver, thus stimulating the expression of collagen I and increase in ROS production [73]. ROS generation by cytochrome P450 2E1 (CYP2E1) in hepatocytes can also induce collagen synthesis and proliferation of HSCs, wich is typically seen in alcoholic liver disease (ALD). ALD usually starts with hepatic steatosis that may progress into fibrosis and subsequent cirrhosis. In the liver, ethanol is mainly metabolized by alcohol dehydrogenases and CYP450 enzymes. This process is associated with several deleterious events, such as the production of ROS, glutathione depletion, lipid peroxidation and increased collagen synthesis [74-75]. Collectively, these mechanisms induce hepatocyte apoptosis, inflammation and the activation of HSCs. CCl_4 is the most widely used hepatotoxin in the study of liver fibrosis and cirrhosis in rodents because hepatic biotransformation of CCl_4 relies on CYP2E1 and yields the trichloromethyl radical, that contribute to an acute phase reaction characterized by necrosis of centrilobular hepatocytes. Also thioacetamide , like CCl_4 , requires metabolic activation to become toxic which is catalysed by CYP450 isoenzymes, results in the formation of thioacetamide sulphur dioxide, which is responsible for hepatic toxicity. DMN and DEN are carcinogenic that are frequently used to experimentally induce liver fibrosis because their biotransformation abundantly induce ROS production. Collectively, these mechanisms induce hepatocyte apoptosis, inflammation and the activation of HSCs. Diet-based models like high-fat diet, methionine-deficient and choline-deficient diet can be used to induce non-alcoholic steatohepatitis in experimental animals; nevertheless, these diet-based models fail to mimic the typical characteristics of the human pathology. Surgery-based models is obtained through bile duct ligation to cause cholestatic injury and periportal biliary fibrosis, nevertheless they are associated to high mortality of the

animals. Finally genetically modified models are rarely developed due to the need of a second stimulus for disease induction.

1.10 In *vitro* models of liver fibrosis

As regard to the *in vitro* models, the primary HSCs derived from healthy liver tissue provide a good reflection of the hepatic *in vivo* situation. When seeded on plastic culture dish, freshly isolated HSCs spontaneously activate and turn into myofibroblast-like cells also occurring during liver fibrosis *in vivo* [76] . Nevertheless, this spontaneous *in vitro* activation triggers a differential gene expression profile in comparison with the *in vivo* counterpart process, which may not reflect the pathophysiological mechanism manifested during liver fibrogenesis. Furthermore, the lifespan of cultured HSCs is limited, which impedes their use. Finally the establishment of human primary HSCs culture is restricted to the lack of biological material. Cell lines appeared as an alternative to primary cells because of their unlimited supply and high inter-laboratory reproducibility of results [77]. A variety of HSC line has been developed. The LI90 cell line was the first human HSC immortalized cell line developed. They display a polygonal shape and a high proliferation rate and produce collagen type I,II, IV, V and VI, fibronectin and α-SMA [78]. Nowadays the most commonly used human HSC cell line is the Lieming XU (LX)-2 , which are created together with the LX-1 line. Both cell lines show a phenotype similar to activated HSCs in vivo and express collagen type I and IV, fibronectin, endoglobin, vimentin, desmin, α-SMA, GFAP, CTGF,surviving,p21, βPDGFR, TGF-β receptor types I

and II, DDR" and Ob-RL [79-80]. Because of their capacity to resemble in vivo HSC activation, LX-2 cells are considered as a model of first choice for investigating the signaling pathways in HSC activation [81]. Another *in vitro* model is based on co-cultures systems, joining 2 cell type has been developed as essential tools to study the interaction between different cell type, which are critical for disease progression [82]. The use of co-cultures consisting of primary hepatocytes and primary HSCS is rare, while HSC cell lines are used to set up co-cultures system with hepatocyte. Recently, the use of co-culture system based on hepatocytes and HSC cell lines demonstrate that the cell-to-cell proximity is of high importance to initiate the fibrotic process induced by fatty accumulation [83]. Regarding this latter issue, stimuli triggering HSC activation can be originating from injured hepatocytes. Hepatocytes are the main source of lipid peroxides in injured liver, thus stimulating the expression of collagen I and increase in ROS production. As a tool for study hepatic physiopathology in alternative to primary hepatocyte are emerging from different cell sources: hepatocytes derived from bone marrow, stem cells, mesenchymal stem cells, hepatoblast, oval cells, embryonic stem cells (ESs) and induced pluripotent stem cells (iPSs) .

1.10.1 Induced Pluripotent Stem Cells for the study and treatment of liver disease

Human pluripotent stem cells (hPSCs) comprise human embryonic stem cells (hESCs), derived from the inner cell mass of the blastocyst of human embryo, and induced pluripotent stem cells (hiPSCs), derived from adult somatic cells through a process of reprogramming with the use of specific factors. hPSCs have distinctive features of pluripotency and self-renewal, meaning that they can proliferate indefinitely in culture while maintaining their characteristic phenotype and the potential to differentiate into all the embryonic germ layers, namely endoderm, mesoderm and ectoderm. Recent and continuous technological advances in the development of stem cells may offer potential applications for human liver disease study, modeling and treatment. These cells allow provide an unlimited supply of disease-specific cells that can be differentiated to the cell type affected by disease. In particular, many studies focused in the last years in the derivation of functional hepatocytes from both hESCs and hiPSCs able to respond to drugs and potentially utilizable for *in vitro* assays [84]. Key feature of mature hepatocytes can be recognized first of all through morphological analysis, presence of bi-nucleated cells and marker expression like hepatic nuclear factor 4α (HNF4α), albumin (ALB) and alpha1-anti-trypsin (A1AT). Metabolic activity is the most important function of hepatocytes, this function is performed by the complex bio-transforming system, which consist of phase I and II metabolizing enzymes such as cytochrome P450. Functionality of hiPSCs-derived hepatocytes can also be assessed through cellular uptake and excretion of indocyanine green (ICG), which is a unique characteristic also of adult primary hepatocytes, as well

as the capacity of accumulate glycogen, revealed by PAS staining. These differentiated cells can be used for disease modelling, drug discovery and autologous cell replacement therapies after genetic correction [85]. In addition iPSCs technology is promising for the development of patient-specific cell therapy. Disease-specific iPSCs lines of inherited metabolic disorders have been generated and their specific disease was successfully modelled [86-89]. In addition molecular mechanisms of other widely known liver disorder such us non-alcoholic and alcoholic liver diseases, viral hepatitis and cirrhosis are currently being investigated [90-98]. Nevertheless, there are still several obstacles and limitations that need to be resolved before the iPSCs technology is routinely used for clinical therapy, for example, information about iPSCs cell lines and protocols of reprogramming and differentiation into hepatocyte-like cells should be optimized and standardized to increase efficiency. Accurate spatio-temporal control of the soluble microenvironment around cell is a critical parameter of this methodology. Extrinsic signal modulation can be used as a parameter for improved germ layer specification and cell differentiation. Functional differentiation of human pluripotent stem cells on a microfluidic platform was previously obtained in our lab [99] proving that accurate spatio-temporal control of the soluble microenvironment around the cell is a critical aspect of this methodology and was achieved through regulation of periodic perfusion frequencies.

1.11 Micro-technologies for cell culture applications

In biological and pharmaceutical research microscale cell cultures are promising supports for high-throughput experimentation in many fields, such as drug screening. Culturing cells at the microscale level allow more precise control of the extracellular microenvironment. Microfluidic platforms combine the advantages of miniaturization and real-time microscopic observation reproducing cell culture conditions that are more physiological than those found in standard *in vitro* systems, such as Petri Dish. Micro-fabrication techniques allow creating devices with characteristic dimensions that are more representative of the physiology *in vivo* [100]. Moreover, they allow to manipulate the cell culture environment in terms of balance between endogenous and exogenous soluble factors and the small dimension of culture leading to mass-limited quantities of molecules. The accurate control of cellular environment is essential to investigate pathway activation and cell behaviour and to control perturbations in the extracellular microenvironment. Microfluidic cell culture platform also combine the advantages of miniaturization and real-time microscopic observation with the ability to pattern cell culture substrates . The control of cellular microenvironment through microfluidic system represent a valuable tool to investigate pathways, controlling phenotype and behavior by monitoring cellular responses to controlled perturbations in the extracellular microenvironment.

Aim of the Thesis

TGF-β signaling pathway is a central regulator of the pathogenesis and the development of various liver diseases, contributing to almost all of the stages of disease progression. Indeed, different liver cells have been recognized to secrete TGF-β ligands and express their related receptors; consequently, these molecules play a crucial role in the progression of liver disease at different stages. The duration of TGF-β signaling appears to be cell type specific, although the exact mechanisms underlying such a variation are still poorly understood. Moreover, different outcomes can be obtained by controlling TGF-β signaling pathway activation, duration and amplitude via intracellular and extracellular mechanisms. Targeting the TGF-β signaling in the pharmacological treatment of liver diseases would constitute a novel approach. In this perspective, it is worth noting that a drug design strategy involving the blockade of TGF-β signaling without high specificity may lead to unknown adverse reactions. Exploring how the temporal dynamic of the pathway is regulated at molecular level is, thus, essential to understand how to control eventual deregulation of the pathway in pathological conditions, such as in liver fibrosis. This work aim to perform a quantitative analysis of the TGF-β pathway activation over time, following specific stimulation in different types of cells, through the development of a microfluidic system. In particular quantitatively assessing TGF-β signaling activity after continuous or pulsatile stimulation to elucidate how ligand exposure could affect gene target expression in different cells. In addition, taking advantage of the microfluidic technology it will be possible to create a microenvironment that

promotes the accumulation of profibrotic or antifibrotic factors in response to profibrotic or antifibrotic stimuli, using different types of cells, known to be involved in hepatic fibrosis. Thanks to this microfluidic system the thrombospondin-1 effect on endogenous TGF-β secreted by liver cells will be analysed. Furthermore we will investigate collagen production mediated by cells of hepatic microenvironment as a functional effect mediated by TGF-β on ECM remodelling. Extracellular matrix modification during fibrotic process could have a specific effect on the endogenous factors expression mediated by hepatocyte to exacerbate or downregulate the fibrotic process. For this latter issues will be developed a microfluidic system for the obtainment of high efficiently hiPSCs derived hepatocyte combining stem cell and cellular matrix derived from fibrotic human liver, for the analysis of soluble secreted molecules from the hepatocyte in contact with the fibrotic matrix.

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CHAPTER II

Material and method

2.1 Microfluidic platform fabrication

Microfluidic platforms are routinely homemade assembled combining polidymethylsiloxane (PDMS), a silicone-derived polymer, and conventional glass slides. Among other elastomeric polymers, PDMS is the most commonly used for cell culture applications because it is non-cytotoxic, autoclavable, gas permeable and optical transparent. Moreover, the flexible nature of PDMS allows the creation of a wide variety of structures ideal for cell culture application, including not only culturing surfaces, but also integrated mechanical valves and pumps. Finally it has low autofluorescence compared to many plastic used in microfabrication, making it an excellent material for imaging application. The technique used to produce PDMS devices suitable for cell culture is mainly composed of two parts: first a re-usable negative master is obtained with soft-litography, then the PDMS layer is prepared through replica molding from the master.

1- SOFT LITOGRAFY

- First the desired structure is designed in CAD (Autocad 2D), and printed on a transparent acetate, creating a photomask.
- Then the photomasks are used to selectively polymerize UV-sensible photoresist with a defined thickness onto a silicon wafer. UV-light intensity, baking time and viscosity of the photoresist have to be take into account in order to achieve selective polymerization of the desired structure. In this project the master was obtained with SU8-2100 negative photoresist.
- Finally, the master need to be treated with hexamethyldisilazane (HDMS) at room temperature for 1 h in order to facilitate the extraction of PDMS mold from silicon wafer. The final result is a re-usable master that can be used for the production of an indefinite number of PDMS replica by replica molding technique

2- REPLICA MOLDING

- A 10:1 solution of PDMS pre-polymer and curing agent (SYlgard 184 from Dow Corning) was cast and cured for 2h at 70°C in an oven.

- Once solidify, PDMS stamps are removed from the master and punched to obtain liquid inlets and outlets. The master is ready to be used again to cast more PDMS stamps.

PDMS layers and glass cover slides can then be assembled and permanently sealed by plasma bonding (Plasma Cleaner, Harrick Plasma, USA). Before cell culture integration, microfluidic platforms were rinsed with isopropanol and sterilized in autoclave. Figure 2.1 summarize the entire material involved in the obtainment of the microfluidic platform.

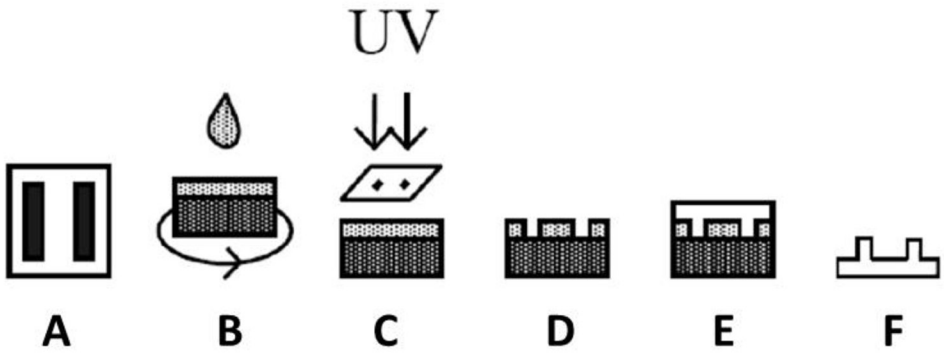

Figure 2.1 Schematic representation of the entire soft-litography process: **A**) photomask realization; **A**) photoresist deposition onto silicon wafer through spin-coating process; **C**) UV exposure ; **D**) master polymerization and removal of non-reticulated material; **E**) PDMS deposition and polymerization; **F**) PDMS mold production.

The final device is therefore a combination of PDMS microfluidic perfusion culture systems and glass as cell culture substrate. In terms of chip-to-world interface, common material choices for tubing and microfluidics that connect the microfluidic perfusion culture system to the macro world include PEEK, Teflon®, Tygon® and silicone. For connecting our microfluidic platform we have used Teflon tubes sterilized at 105°C for 30 minutes In this work was developed microfluidic devices containing 3 or 10 microchannel (18mm long,1.5 mm wide and 0.2 mm high) working in parallel. Photomask in figure 2.2a was designed by Dott. Michielin (University of Padova, department of Industrial Engineering),

printed onto a transparent polymer sheet and photolitographically patterned onto a silicon wafer with SU8-2100 negative photoresist , to obtain a final thickness of 200 μm, according to manufacturer instructions. The master was then treated with HDMS as previously mentioned and the pre-polymer solution and curing agent was cast and cured. Finally PDMS mold was cut and peeled off, punched to obtain liquid inlets and outlets and sealed to a previously cleaned glass slide by plasma bonding (figure 2.2b). Following this procedure 10 PDMS well of 3 mm of high and diameters was sealed on microchamber outlet to create a medium reservoirs. Figure 2.3 represent the entire realization of the microfluidic platform.

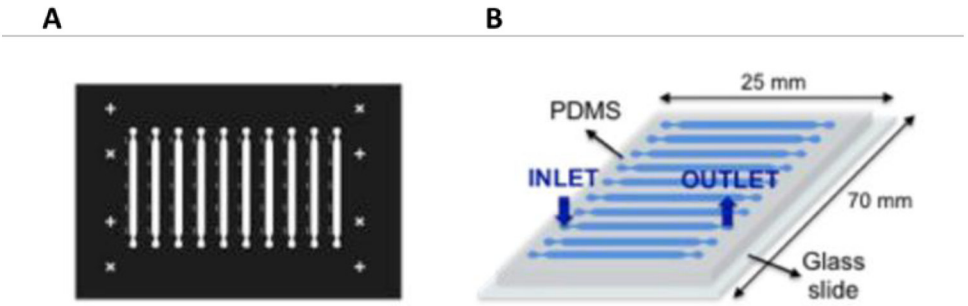

Figure 2.2 Microfluidic device for cell cultures integration. (A) Photomask with microfluidic channels. (B) Microfluidic chip with 10 independent channels.

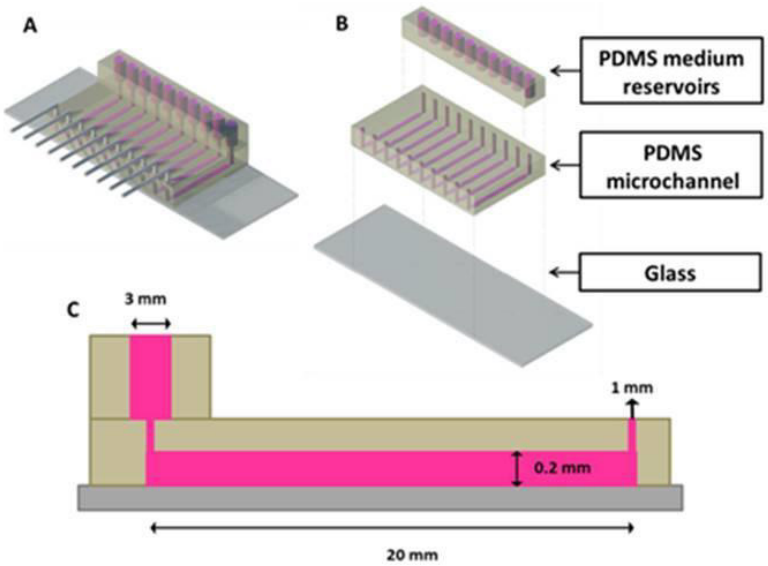

Figure 2.3 **S**chematic representation of microfluidic platform with 10 indipendent microchannel. Three-dimensional representation on microfluidic platform that can be connected with other devices (A). Schematic representation of the three layers that composed microfluidic platform (B). Lateral section in which are indicated the 3 layers with the relatives measures (C).

2.2 Decellularized matrix integration it the microfluidic platform

For the production of the microfluidic platform containing decellularized matrix , Superfrost glass were first treated with UV light for 15 minute and subsequently treated with polylysin 1µg/ml for one hour and maintained in sterility condition. Decellularized liver matrix derived from healthy donor or cirrotic patient were obtained from University college of London and maintained in PBS solution at 4 °C until use. Cylindrical sample of 1 mm of diameter were punched and freeze with liquid nitrogen. Cryosection of 40 µm were obtained at cryostat (Cryostat Leica 1860) and put on glass slide.

2.3 Cell culture

2.3.1 Human Cheratinocyte HaCaT

Immortalized human cheratinocyte HaCaT, stably transfected with $(CAGA)_{12}$ inducible TGF-β1 plasmid luciferase reporter, were maintained in DMEM (Dulbecco's Modified Eagle Medium) 10% FBS (Fetal Bovine Serum), 1% non-essential amino acid (NEAA), 1% pen-strep (100U/ml penicillin, 100 mg/ml streptomycin). Cells were routinely expanded in T75 culture flask (Falcon) and seeded in multiwell plate of different surface (from 8.87 to 0.29 cm^2) and also in microfluidic chamber at confluency to perform the experiments. Every single microfluidic chamber was coated with 12 µl matrigel® 0.5% in DMEM for 45 minute at room temperature. Cells were then seeded in the microfluidic platform at a density of 10^3 cells/mm^2 using a volume of 12 µl. In microfluidics, culture media were changed every 12 hours using 12 µl per chamber, while in multiwell plates the media were replaced once a day.

2.3.2 Human Embrionic Stem Cells

Human Embrionic cell line (HES-2), derived from the inner mass of blastocyst (obtained from National Stem Cell Bank, Madison, WI) were cultured in TeSR-E8 (Stemcell Technologies) in multiwell plates coated with 0.5% MRF (Matrigel Reduced Factor) (BD®) and passaged with 0.5 mM EDTA (Life Technologies).

Cells were seeded in the microfluidic platform coated with 0.5% MRF at a density of 2 x 10^3 cells/mm^2. Medium was change every 12 hours to avoid the lack of nutrients.

2.3.3 Human primary hepatocyte

Human primary hepatocyte were obtained from Bioreclamationivt S.p.A. Cells were derived from whole human liver not suitable for transplantation and preserved in cryovials. Once defrosted cells can be maintained in culture for five days at most and cannot be refrozen. Cells were cultured in their specific commercial media (unknown composition) in multiwell plates coated with collagen I 5 μg/cm^2. These cells were used as a control for the differentiation of human pluripotent stem cells in hepatic-like cells.

2.3.4 HepG2 cell culture

Cell line HepG2 , derived from hepatic carcinoma , were firstly used as an *in vitro* model of the human hepatocyte, thanks to the ability of secretion of plasmatic proteins. Nevertheless these cells have an aberrant number of chromosomes (55) and doesn't faithfully represents the complexity of the hepatocyte. Cells were cultured in RPMI 1640 (Life Technology) with 10% FBS. Cells were seeded in microfluidic platform at a density of 2 x 10^3 cells/mm^2.

2.3.5 Human Stellate Cells LX-2

Immortalized Stellate Cells LX-2, obtained from the laboratory of Prof. Marra in the department of Experimental Medicine in the University of Firenze were maintained in culture in DMEM , 10% FBS. LX-2 represent a first choice line for the study of hepatic fibrotic process. Cells were seeded in the microfluidic platform at a density of 2 x 10^3 cells/mm^2.

2.3.6 Human macrophages derived from monocyte

Human macrophages derived from monocyte in human blood were obtained from Dott. Trenti using a protocol validated in the laboratory of Prof. Bolego (University of Padova – Pharmacology Department). Monocyte were cultured for one week in RPMI 1640 (Life Technology) using M-CSF and FBS 10%. After 7 days macrophages were activated using LPS/IFNγ or IL4/IL13 for 48 hours to respectively produce M1 and M2 phenotype. Macrophages were passed from the well plate to the microfluidic platform at a density of 2 x 10^3 cells/mm^2 in RPMI 1640 in 10% FBS.

2.4 Cell cultures integration into microfluidic devices

Cells in microfluidic system are infused through an external inlet. The seeding flow rate employed must be relatively low not to compromise cell viability. However a flow too slow may produce a non-uniformity in the final cell density. A critical issue in microfluidic culture is the good cell attachment during cell seeding. Especially when using high surface-area-to-volume ratio is essential to ensure a sufficient time window to allow cell attachment without nutrient and oxygen depletion, before performing the first medium change. The duration of this time of attachment may vary depending on cell type, cell density, cell substrate and culturing media; hESCs tipically required 4 hours for static. Cellular dynamic inside the microfluidic device can be monitored via imaging or fluidic integration. Imaging using exogenous fluorophores or luminescence construct are ideal to collect data from microdevices because it enables dynamic measurements In particular it was used both fluorescence to analyse the expression of specific marker and luminescence to perform functional analysis on pathway activation. This microscale measurement allow to reduce the number of cell needed and allow the development of ultra-sensitive miniaturized analytical techniques for detecting molecules secreted by a very small number of cell. Cell integration inside the microfluidic devices can be reversible or irreversible. The first system is particular important when reuse of microfluidic devices is important and also when is needful to remove microfluidic device from the bottom glass slide at the end of process performed. In the experiments also reversible and irreversible microfluidic platform are used depending on different application. Reversible

integration was used for producing cell substrate adhesion based on extracellular matrix from donor tissue, while irreversible integration was used when cells were seeded on uncoated glass or on protein coating. Reversible integration is also useful in fast and repeated experiments, in order to reduce the time and cost. The use of this technology give the possibility to integrate different substrate inside the microfluidic platform and also seeding cells in a different step of the process.

Irreversible integration allows the direct insertion of cells inside microfluidic chamber followed by long term culture and differentiation. Glass is often used as a cell culture substrate because it can be permanently bonded to the PDMS microfluidic chip, but it can present many issues to cell attachment. The cell substrate material allow cell attachment via adhesive proteins. Uncoated glass normally support the culture of fibroblast and cheratinocyte , but not allow the adhesion of hESCs. In this case we pre-incubate glass with matrigel reduced factor (MRF) BD® for 45 minute at room temperature. Thus , surface modification of cell substrates to facilitate cell attachment can be critical in microfluidic perfusion culture. Reversible integration was used to create a microculture chamber in which cells can be seeded on extracellular matrix derived from decellularization process. Decellularized human liver matrix obtained from Institute of Child Health, University College London, was frozen in liquid nitrogen, cryosectioned at 40 µm slice and deposed on a polylysine-treated glass surface. The PDMS structure was positioned on top of the glass slide without plasma treatment and at the end of the process cells was integrated using the same protocol of irreversible integration.

2.5 Microfluidic co-culture system

Co-culture experiment were perform in order to evaluate the possibility to incubate different cell type sources in a same microfluidic chamber maintaining the two culture without any physical interaction but in the same culture media: HaCaT and HES-2 cells were incubated separately respectively on the bottom and on the top of the microfluidic platform. Microfluidic platform with a specific high of 200 μm were incubated with 2% MRF at room temperature for 45 minute on the side of glass and for 45 minute on the side of PDMS. HaCaT at the density of 10^3 cells/mm^2 were seeded in the microfluidic platform on the glass side. After 12 hours HES-2 cells were seeded at the density of 600 cells/mm^2 and microfluidic chip was rotate of 180° in z dimension to adhere the cells on the PDMS side. After more 12 hours microfluidic chip was rotate on the original position (figure 2.4)

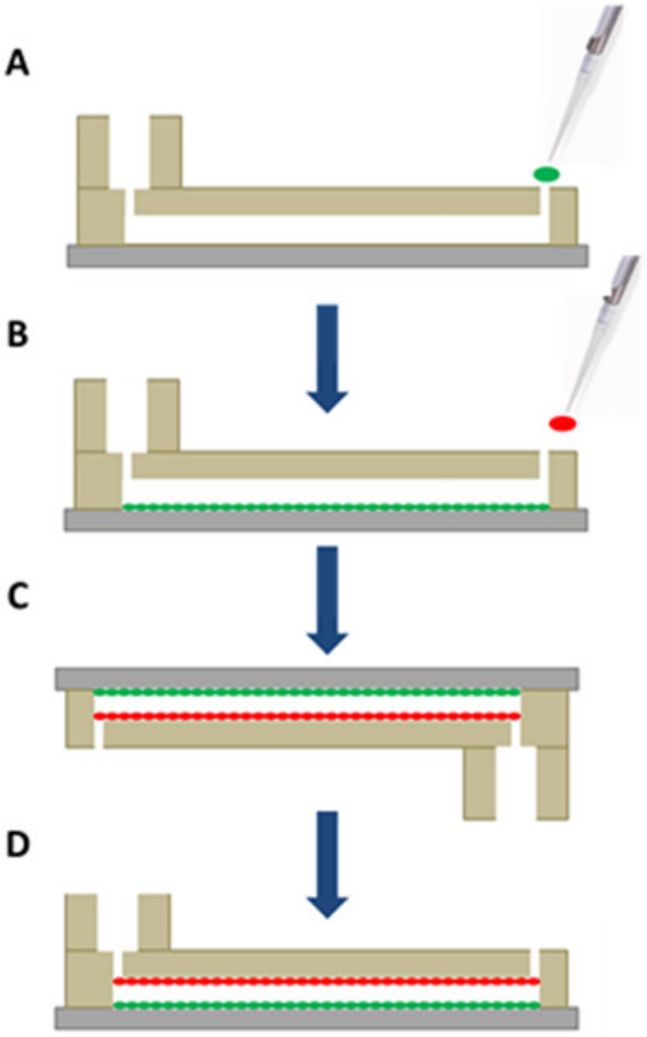

Figure 2.4 Schematic representation of cell co-culture integration in microfluidic platform. Injection and seeding of HaCaT cells on glass surface (A) . Injection of HES-2 cells (B) and seeding on PDMS after 180° rotation of platform in z dimension (C). Obtainment of the HES-2 cells of the top of the surface after 180° rotation of platform in z dimension (D).

2.6 Obtainment of conditioned media in microfluidic system

Microfluidic system allow the concentration of soluble factors secreted by the cells at a level impossible to obtain in traditional well plate and also increase the autocrine stimulation of the resident cells. Before the collection of conditioned medium cells were maintained for 12 hours in absence of serum to remove every not defined component in the culture medium. After this period media were changed and collected at the end of the following 48 hours. Conditioned media were then also used as it is or thermally treated for 5 minute at 95°C to activate the TGF-β produced as a latent complex.

2.7 Immunofluorescence

Immunofluorescence staining was used to identify specific target on cell samples. The technique is based on antibody specificity towards specific antigens and allow to identify the expression and distribution of molecules of interest. The following analysis were conducted through indirect immunofluorescence technique to amplify the signal and using a single antibody specific for more primary. Solution and antibody used in the listed in table 4A and 4B.

Fixing solution	paraformaldehyde 4%
Washing solution (PBS)	PBS (-Ca2+/Mg2+)1X
Washing solution (PBST)	PBS (-Ca2+/Mg2+)1X + TritonX-100 0,1%
Blocking solution	PBS (-Ca2+/Mg2+)1X + Horse Serum scomplementatated 5% + TritonX-100 0,1%
Primary antibody	Diluted in blocking solution
Secondary antibody	Diluted in blocking solution
Nuclear marker	DAPI 1:1000 in PBST

Table 3 Immunostaining solution.

A

Antiody	**Specificity**	**Dilution**	**Incubation**	**Host**	**Product number**
OCT4	Human, Mouse	1:200	Overnigth	Mouse	Santa cruz / sc-5279
FOXA2	Human, Mouse	1:400	Overnight	Rabbit	Cell Signaling/D56D6
SOX17	Human	1:200	Overnight	Goat	R&D/AF1924
HNF4a	Human	1:100	Overnight	Mouse	Santa Cruz/ sc-8987
A1AT	Human	1:00	overnight	Rabbit	Dako/A0012
ZO-1	Human	1:100	Overnight	Rabbit	GTX108627
CYP3A4	Human	1:00	overnight	Mouse	Santa cruz/ 365415
AFP	Human	1:100	Overnight	Rabbit	Sigma/A8452
CK-18	Human	1:100	Overnight	Rabbit	Genetex/105624
F-ACTIN	Human	1:100	Overnight	Rabbit	Santa cruz/130935
E-CADHERIN	Human	1:50	Overnight	Rabbit	Genetex/100443
SMAD7	Human	1:100 IF 1:250 WB	Overnight	Rabbit	Sigma_SAB4200345
COLLAGEN I	Human	1:100	Overnight	Rabbit	Fitzgerald-70R-CR009x
COLLAGEN VI	Human	1:100	Overnight	Mouse	Millipore / MAB1944

B

Specificity	**Conjugated fluorophore**	**Dilution**	**Incubation**	**Host**	**Product number**
Goat	Cy2	1:200	1 hour 37°C	Donkey	Invitrogen
Mouse	Alexa 594	1:200	1 hour 37°C	Goat	Invitrogen / A11005
Rabbit	Alexa 594	1:200	1 hour 37°C	Mouse	Invitrogen / A11012

Table 4 Primary (A)and secondary(B) antibody used for immunostaining.

2. 8 Real Time PCR

Gene expression analysis were conducted through qPCR (*Real Time polymerase Chain Reaction*). RNA extraction was mediated by *iScript* reagent (Bio-Rad), maintained for 1 minute in contact with the cells. The RNA extracted was maintained at -20 °C. Reverse transcription was performed with High Capacity cDNA Reverse Transcription Kit (Applied Biosystem). Reaction component and retrotranscription protocol are described in table 5.

Component	**Volume per reaction**
10X RT Buffer	2.0 mL
25X dNTP Mix (100 mM)	0.8 mL
10X RT Random Primers	2.0 mL
MultiScribeTM Reverse Transcriptase	1.0 mL
H2O RNasi/DNasi-free	4.2 mL
RNA total solution(fino a 2 mg)	10.0 mL
total reaction Volume	20.0 mL

	Step 1	**Step 2**	**Step 3**	**Step 4**
Temperature [°C]	25	37	85	4
Time	10 min	120 min	5 min	∞

Table 5. Composition of reaction mix (A) and retrotranscription protocol (B).

In this work, gene expression is valuated through Real Time PCR with TaqMan probes. TaqMan system is composed by Forward and Reverse Primers combined with a third cDNA probe functionalized with a fluorophore at 5' end, and a quencher at 3' end. The probe is designed to bind the target gene between

Forward and Reverse Primer. Thanks to the exonuclease activity of the Taq polymerase, during the elongation step of the PCR process, the quencher is separated from the fluorophore and the fluorescent signal can be detected. All the experiment were conducted by TaqMan Gene Expression Assay (Applied Biosystem) using AmpliTaq Gold® DNA Polymerase and TaqMan probe conjugated with 6-FAM at 5' and Non Fluorescent Quencher at 3'. All the reaction were conducted using 96 well plate with 10 µl of volume reaction. As a gene expression control were used glyceraldehyde-3-phosphate dehydrogenase (GAPDH), conjugated with fluorophore VIC. All the data were expressed as a relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The component of reaction mix were indicated in table 6 and the TaqMan probe are listed in table 7. Data obtained were analysed with ABI prism 7000 SDS Software.

Component	**Volume per reaction**
20X TaqMan® Gene Expression Assays	0.5 ml
20X TaqMan® Gene Expression Master Mix	5 ml
cDNA template (from 1 to 100 ng)	2 ml
H2O Rnasi/Dnasi-free	2.5 ml
Total volume per reaction	10 ml

Stage	**Hold step cycle (40)**			
Temperature [°C]	50	95	95	60
Time	2 min	10 min	5 sec	1 min

Table 6. Composition of the reaction mix and protocol for Real Time PCR.

Gene	Species	Transcript	ID number Termofisher
SMAD7	Human	4 RefSeqs (NM)	Hs00998193_m1
SERPINE1 (PAI1)	Human	1 RefSeq (NM)	Hs01126606_m1
SNAI1 (SNAIL1)	Human	1 RefSeq (NM)	Hs00195591_m1
ALPHA1 ANTI-TRIPSIN (A1AT)	Human	11 RefSeq (NM)	Hs00165475_m1
FOXA2	Human	2 RefSeq (NM)	Hs00232764_m1
ALPHAFETOPROTEINA (AFP)	Human	1 RefSeq (NM)	Hs00173490_m1

Table 7. TaqMan probe used in Real Time experiment

2.9 Gene Silencing

Gene silencing was performed transfecting HaCaT cells with SMAD7 siRNA #4392420 (Thermofisher) and negative control #4390843 (Thermofisher) using Stemfect RNA transfection Kit (Stemgent). Transfection protocol was optimized on 96 well. HaCaT cells were seeded at a density of 2 x 10^3 cells/mm^2 to reach a confluence of 70% in 24 hours. Fresh culture medium without serum was added 2 hours before the transfection using 150 µl. Transfection protocol was then performed following the manufacturer's instructions. 0.08 µl of Stemfect Transfection Reagent were added to 7.5 µl of Transfection Buffer. At the same time 50 pmoles of siRNA were added at the same volume of buffer and the two solutions were mixed and incubated for 15 minute at room temperature. After this time 15 µl of solution were added to every single well. Silencing protocol was validated through protein expression analysis 72 hours after the transfection.

2.10 Western Blot

Protein expression was analysed through Western Blot. Cells in traditional well culture or microfluidic system were lysate using Ripa Buffer. SDS page was performed using gel NuPage 4-12% Bis-Tris (Invitrogen) and the electrophoresis was performed for 1 hour and 45 minute at 400 mA in NuPage transfer buffer (Thermofisher). Proteins were then transferred on PVDF membrane (Invitrogen) for 2 hours (45 V, 400 mA) in NuPage transfer buffer (Thermofisher). The membrane was blocked using 5% Blotting-grade Bloker (Bio-Rad) for 45 minute at room temperature and incubated with primary antibody Overnight at 4°C. The membrane was washed 3 times for 5 minute each in TTBS and then incubated with the proper HRP-conjugated secondary antibody. After additional 3 wash proteins were visualized by enhanced chemiluminescence using Novex ECL HRP (Invitrogen). Images were collected using ImageQuant LAS 4000 mini and quantify using software ImageJ.

2.11 Luciferase reporter activation on live cells in a microfluidic system

Functional study on pathway activation in a cellular model was performed using luciferase as a reporter protein in transgenic keratinocyte cell line (HaCaT) containing a TGF-β inducible $(CAGA)_{12}$ luciferase construct. By acquiring luminescence production directly mediated by cells it was possible to monitor the genic expression of a gene of interest in a continuous manner. The luciferase

reporter gene is obtained fusing the putative regulatory elements to a reporter gene and monitoring the amount of the reporter protein expressed. Because reporter expression is under control of the fused genetic elements, reporter expression is directly correlated with the activity of the regulatory elements. The luminescence reaction is based on luciferase enzyme and all the data collection is based on luminescence production during the time of the entire experiment; for this reason is essential to take in account all the parameter and the limiting agent of the reaction. For catalysing luminescent reaction on live cell luciferin substrate has to penetrate inside the cells and its concentration has to be in large excess to avoid the occurrence of substrate depletion during the long term acquisition. Moreover other chemical substrates have not to result as a limiting reagent in order to convert chemical energy from ATP to light reacting luciferin, adenosine triphosphate (ATP) and oxygen (O_2). The final products of this reaction are oxidized luciferin ("oxyluciferin"), adenosine monophosphate (AMP), inorganic pyrophosphate (PPi), carbon dioxide (CO_2) and light. Photons emitted from bioluminescence reaction can be measured by an high sensitive detectors. Luminescence assays were performed using two different methods: on cell lysate or cells in culture. In the first case reporter cells were maintained in 12 multiwell plate until reaching 80% of confluency and then maintained in DMEM41965 (life Technologies) without serum for 12 hours. Cells were then treated with media containing TGF-β for 7 hours, lysate and frozen overnight. Cell lysate was then mixed for 30 second and centrifugated at maximum speed for 10 minute at 4°C. 40 µl of supernatant were transferred on MW96 luciferase white plate (FALCON 353296) and 5 µl were used for the protein quantification through bradford assay. In every single luciferase experiment we used a positive luminescence control by

adding TGF-β (Peprotech) 1 ng/ml to the medium and a negative control by adding 10 μM TGF-βR1 inhibitor SB431542 (Stem Gent). The inducible expression of luciferase results in this case as a functional effect of the TGF-β pathway activation as a process that beginning from the binding of the ligand to receptor until transcriptional activation of target genes and protein translation. To assess the reporter construct activation following TGF-β stimulation, we optimized the luminescence assay on cell lysate by varying the stimulation time with TGF-β 1 ng/ml. Luminescence signal revelation on live cells into the microfluidic platform was performed using the same condition as the lysate assay, with the exception of luciferin, that is essential to be in the functional chemical form to penetrate inside the live cells to become a substrate of the luminescence reaction. In this case we use 1 mM D-Luciferin free acid (Duchefa). Luminesce on live cells was detected by Vivo Vision IVIS 100 Series (XENOGEN). The quantification of the signal was take in account the possibility to downscale from multiwell plate to microfluidic system, for this reason we analyse the luminescent signal from different number of cell plated on different multiwell plate maintaining the same volume to surface ratio. The quantification of signal was expressed in counts, that is an index of number of light fotons expressed in time unit, on surface unit (mm^2).

2.12 Live&Dead staining

Cell survival inside the microfluidic platform was evaluated using the LIVE/DEAD® system (Molecular Probes, Invitrogen), a two-colour fluorescence assay based on membrane integrity and esterase activity. Live cells contain ubiquitous intracellular esterases, which can convert the non-fluorescent cell-permeant calcein AM to the intensely fluorescent calcein; this latter is retained within live cells and emits green fluorescence (ex/em~495 nm/~515 nm). On the other hand, ethidium homodimer-1 enters only cells with damaged membranes and undergoes a 40-fold enhancement of fluorescence upon binding to nucleic acids, thereby producing a bright red fluorescence (ex/em ~495/~635 nm). Assays were carried out following the manufacturer's instructions, by incubating the scaffolds with a solution of calcein (3μM) and ethidium homodimer-1 (2μM) in PBS for 45 min in the dark. Labelled cells were observed using a Leica DMI 6000B fluorescence microscope. The percentage of dead cells within each microfluidic channel was calculated by scoring 3 randomly taken optical section.

2.13 Periodic Acid Staining

Periodic Acid Staining (PAS) was performed to analyse the glycogen storage level in primary hepatocytes and hiPSCs-derived hepatocyte-like cells. Cells were fixed on glass surface using paraformaldeyde 4% for 10 minute. After two wash with milliQ water cells are incubated 5 minute with periodic acid, 15 minute with

Schiff reagent and 1 minute with Hematoxilin performing 3 wash with milliQ water between every step. Cells were observed using a Leica DMI 6000B.

2.14 CytocromeP450-3A4 assay

P450-Glo™ assay provide a luminescent method to measure cytochrome Cytocrome P450 activity. The P450-Glo™ Substrates are CYP enzyme substrates that are pro-luciferins, that can be converted by CYP enzymes to luciferin products. Luciferin is also detected in a second reaction with the Luciferin Detection Reagent . The amount of light produces in the second reaction is proportional to CYP activity. Cells were treated with ryfampicin (15-100 μm) for 12 hours and then exposed to luciferin-6'-pentafluor-benzil-etere (PFBE) for 20 minute. This substrate is metabolized from CYP3A4 releasing free luciferin. Adding luciferase contained in the Promega Buffer allow to reveal luminescence signal.

2.15 Total collagen assay

Total quantification of collagen produced by HepG2 and stellate cells LX-2 was performed by QuickZyme total collagen assay kit (Quickzyme Biosciences) following the manufacturer's' instruction. Collagen quantification is based on the detection of hydroxyproline, a non-proteinogenic amino acid, which in mammals

occurs in elastin and collagen. Hydroxyproline is formed post-translationally from specific proline residues by action of the enzyme prolylhydroxylase. The measurement of collagen is started by complete hydrolysis of cell lysate in 6M HCl at 95°C. Using a specific reagent that bind hydroxyproline is possible to reveal the increase of 570 nm signal directly correlated to the amount of collagen.

2.16 Pluripotent Stem Cell differentiation in hepatic-like cells

Differentiation protocol of pluripotent Stem Cells into hepatic-like cells takes 17 days and is divided in 3 distinct step: 1) endoderm induction, 2) hepatic differentiation and 3) hepatic maturation. Endoderm was induced with RPMI-B27-insulin, PI-103 50 mM, 100 ng/ml Activin-A, 50 ng/ml Wnt3a and 1% penicillin-streptomycin form 1 day and then changed to RPMI+B27 100 ng/ml ActivinA, 50 ng/ml Wnt3a for 2 more days. Medium was changed to KO-DMEM with 20% KSR, 1 mM L-glutamine, 1% NEAA, 0.1 mM β-mercaptoethanol, 1% DMSO (Sigma-Aldrich) and 1% penicillin-streptomycin for 5 days. Hepatic cells were maturated with L15 medium (Sigma-Aldrich) supplemented with 8.3 % FBS , 8.3% tryptose phosphate broth (Life Technologies), 10 ng/ml hepatocyte growth factor and 20 ng/ml oncostatin M (both from R&D) for 9 days. Differentiation protocol was also applied on hiPSCs growth on hepatic decellularized matrix from healty donor and cirrotic patients. Endoderm induction was evaluated through the presence of specific marker like FOXA2 and SOX17. From the day 4th to 8th is it possible to reveal the typical conformation of the hepatocytes and the production of hepatic marker like Hepatic Nuclear Factor α (HNF4a).

Complete hepatic maturation is obtained from the day nine to the day 17. The most important maturation marker analysed are the following: Alphafetoprotein (AFP), CK-18, E-cadherin, alpha-1-antitrypsin, ZO-1, F-actin, albumin and CYP3A4.

2.17 Statistical analysis

For statistical analyses, single pairwise comparisons were analyzed using Student's t-test with $P \leq 0.05$ (*) or $P \leq 0.01$ (**) indicating significance. Multiple comparisons were performed by one-way ANOVA with Tukey post-test, with $P \leq 0.05$ (*) or $P \leq 0.01$ (**) indicating significance. Results was expressed as mean ± SD of at least three independent experiments.

CHAPTER III

Real time analysis of TGF-β pathway activation in a microfluidic platform

One way to achieve diversity in signaling outcomes is by controlling signal duration. In this chapter it was analysed the effect of continuous or pulsatile stimulation with TGF-β on signaling dynamic. In particular it was used a microfluidic system to perform a Real Time analysis of TGF-β pathway activation in a TGF-β reporter cell line and also analyze how the signaling pathway amplitude and duration is associated to endogenous target gene expression. In particular it was focused the attention on SMAD7 , revealing a specific dynamic that is indipendent form TGF-β stimulation profile.

3.1 Luciferase reporter activation on live cells in a microfluidic system

Functional study on pathway activation in a cellular model was made up using luciferase as a reporter protein in transgenic reporter keratinocyte cell line (HaCaT) containing a TGF-β inducible $(CAGA)_{12}$ luciferase construct. By acquiring luminescence production directly mediated by cells it was possible to monitor the genic expression of a gene of interest in a continuous manner. The luciferase reporter gene is obtained fusing the putative regulatory elements to a

reporter gene and monitoring the amount of the reporter protein expressed. Luciferase expression, is directly correlated with the activity of the regulatory elements. The luminescence reaction is based on luciferase enzyme and all the data collection is based on luminescence production during the time of the entire experiment; for this reason is essential to take in account the presence of limiting reagents in the reaction. For catalysing luminescent reaction on live cell, luciferin substrate have to penetrate inside the cells and its concentration have to be in large excess to avoid the occurrence of substrate depletion during the long term acquisition. Moreover other chemical substrate have not to result as a limiting reagent in order to convert chemical energy from ATP to light reacting luciferin, adenosine triphosphate (ATP) and oxygen (O_2). The final products of this reaction are oxidized luciferin ("oxyluciferin"), adenosine monophosphate (AMP), inorganic pyrophosphate (PPi), carbon dioxide (CO_2) and light (fig 3.1). Photons emitted from bioluminescence reaction can be measured by a high sensitive detectors.

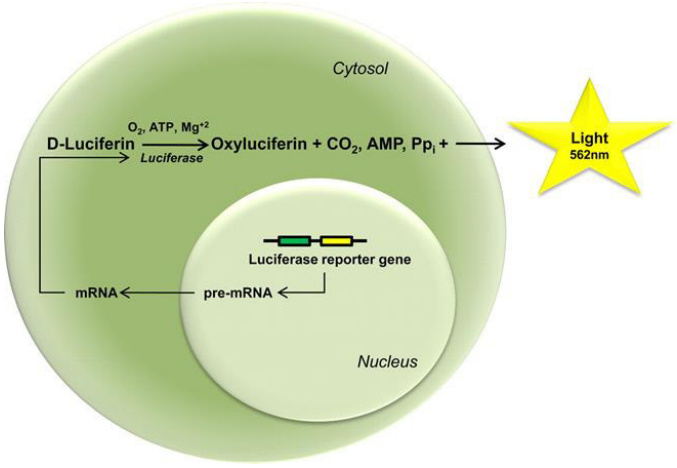

Figure 3.1 Schematic representation of luciferase reporter gene activation and bioluminescence reaction.

The inducible expression of luciferase result in this case as a functional effect of the TGF-β pathway activation as a process that begins with the binding of the ligand to receptor until transcriptional activation of target genes and protein translation. To assess the reporter construct activation following TGF-β stimulation it was optimized the luminescence assay on cell lysate by varying the stimulation time with TGF-β 1 ng/ml. Figure 3.2 shows the luminescence signal normalized on the total protein content based on luciferase accumulation during a period of time ranging from 1.5 to 12 hours. The result of this experiment showed a peak of signal at 7.5 hours. Thus, this time point was chosen to perform the acquisition of the luminescence signal.

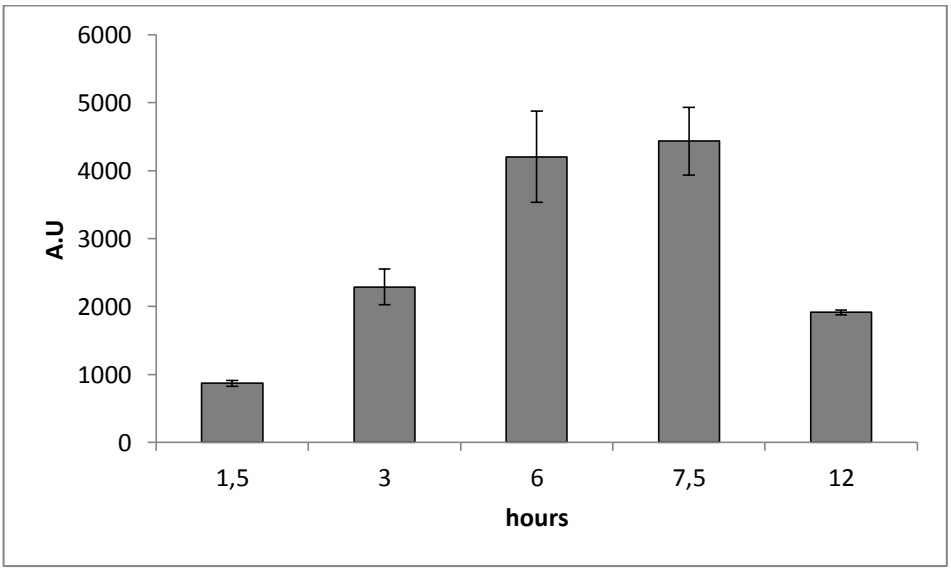

Figure 3.2 Luminescence signal based on luciferase accumulation in HaCaT cell lysate at different time interval after TGF-β (1 ng/ml) stimulation. Data are express as mean of 3 indipendent experiment ± standard deviation.

The detection of the luminescence signal on live cells within the microfluidic platform was performed using the same conditions as for the lysate assay, with the exception of luciferin, that is essential to be in the functional chemical form to penetrate inside the live cells to become a substrate of the luminescence reaction and have to be added at the same time with TGF-β at the beginning of the experiment. It was also investigated the possibility to downscale from multiwell plate to microfluidic system. For this reason was analysed the luminescent signal from different number of cells plated on different multiwell plate, maintaining the same volume to surface ratio. The quantification of signal was expressed in counts, an index of number of light fotons expressed in time unit, on surface unit (mm^2). In figure 3.3 we observed that the downscale of the system occurs without affecting the sensibility of the detection in different multiwell plate and also in the microfluidic system.

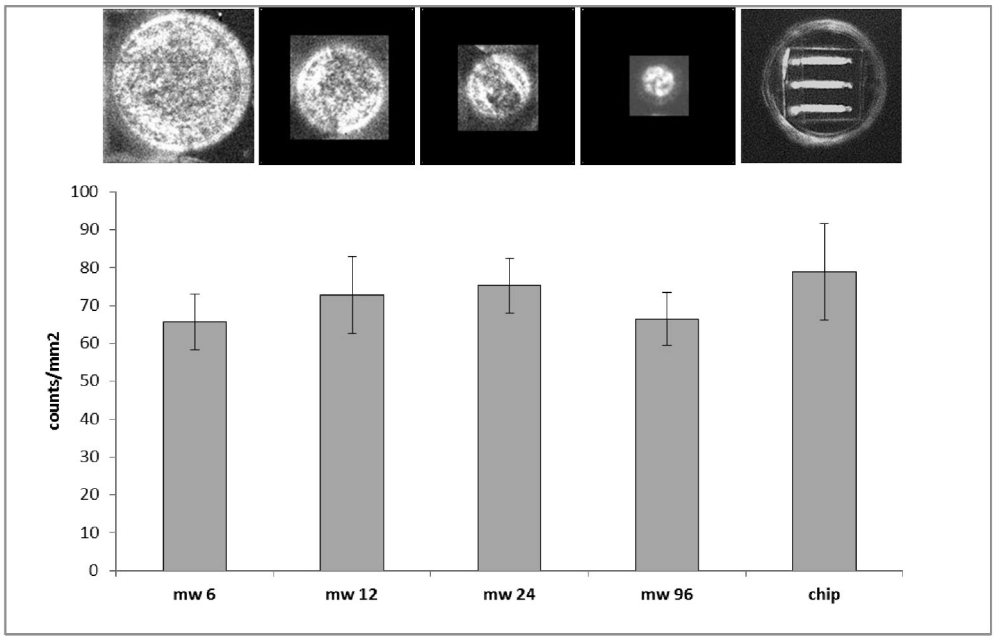

Figure 3.3 Quantification of luminescence signal on surface unit based on luciferase accumulation in HaCaT live cells stimulated with TGF-β (1 ng/ml) seeded on different surface, maintaining the same cell concentration. Data are express as mean of 3 indipendent experiment ± standard deviation.

3.1.1 Microfluidic platform for real-time analysis

The microfluidic systems fabricated for luminescence analysis (figure 3.4a) allow to transfer specific amount of medium between two different microchamber. Connecting two different chip using Teflon tubes allow the possibility to transfer (figure 3.4 b) specific amount of media between the micro-chamber Medium delivery could be accurately controlled by adding specific amount of medium to the inlet of the micro-chamber. The transfer of medium by capillarity occurs without any external pump (figure 3.1c). It was possible to integrate two different cells types: hESCs , that secrete TGF-β in the chamber upstream and HaCaT TGF-β luciferase reporter in the chamber downstream. The seeding uniformity of HaCaT and hESCs cells was also evaluated (figure 3.5). Based on microfluidic platform design, we also developed a strategy to transfer conditioned media from two different micro-chamber by adding micro-volume of media upstream the system and revealing the signaling downstream (figure 3.6).

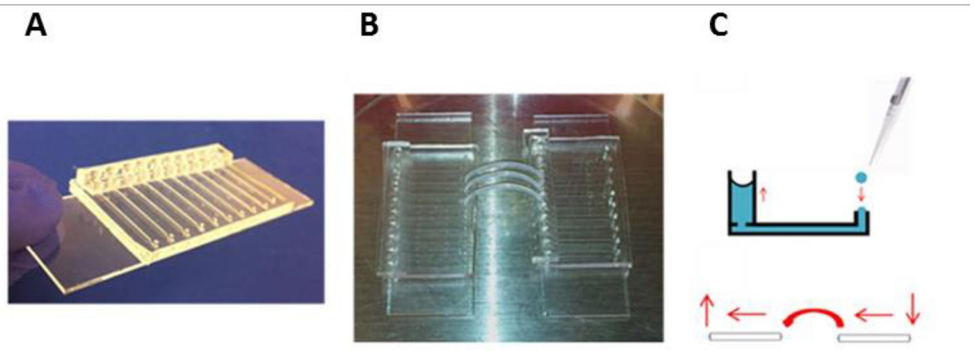

Figure 3.4 A) microfluidic platform , (**A**) microfluidic platform connection with Teflon tube (**C**) mechanism of medium transfer across two micro-chamber.

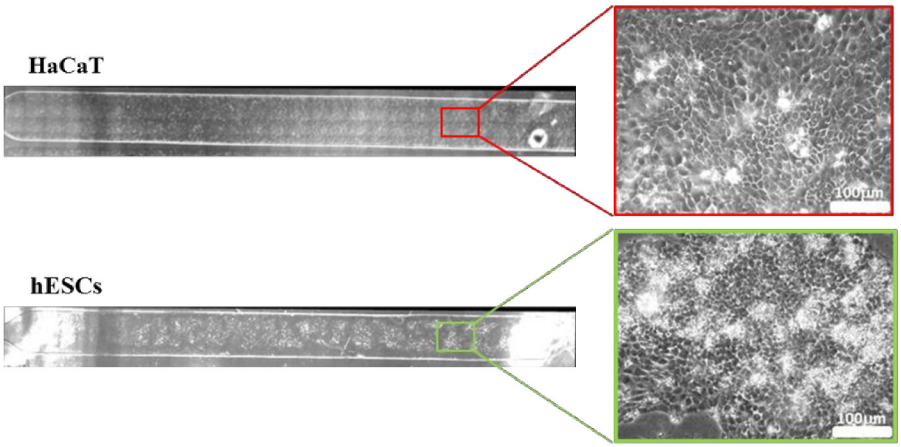

Figure 3.5 Integration of HaCaT and hESCs inside the microfluidic platform, respectively seeded on MRF 0.5% coated glass.

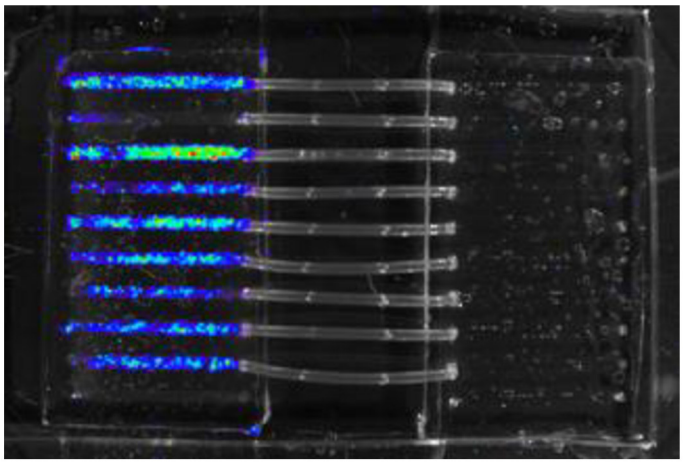

Figure 3.6 TGF-β medium transfer strategy though two different micro-chamber by adding media upstream in the right chamber and reading luminescence signal on HaCaT reporter cell line downstream in the left micro-chamber.

Co-culture experiments were also performed in order to evaluate the possibility to incubate different cell types sources in a same microfluidic chamber maintaining the two culture without any mechanical interaction but in the same culture media: HaCaT and hESCs were incubated separately respectively on the bottom and on the top of the microfluidic platform after 12 hours from the first seeding. Live&Dead staining performed after 48 h from the second seeding show an

almost complete cell viability and cell type separation on two different surface (figure 3.7).

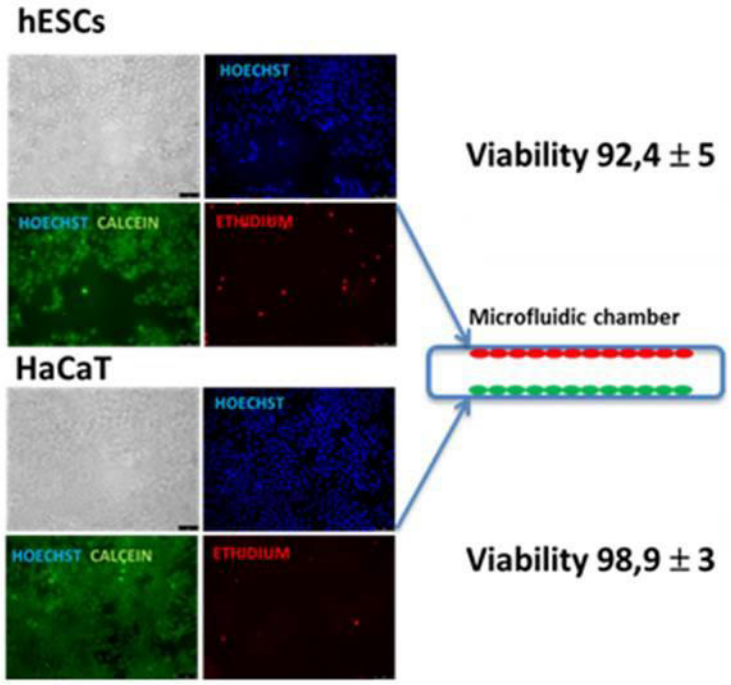

Figure 3.7 Live&Dead assay performed on co-culture of hESCs and HaCaT seeded respectively on the top and on the bottom in a single microfluidic chamber. Data are express as mean of 3 indipendent experiment ± standard deviation.

To assess the possibility to obtain long term luminescence acquisition on live cells LIVE&DEAD assay was performed in order to assess cell viability of HaCaT cells during this period of time. The assay resulted in a cell viability of 95 ± 6% (figure 3.8), which confirms the possibility to acquiring luminescence for a long period of time with this system.

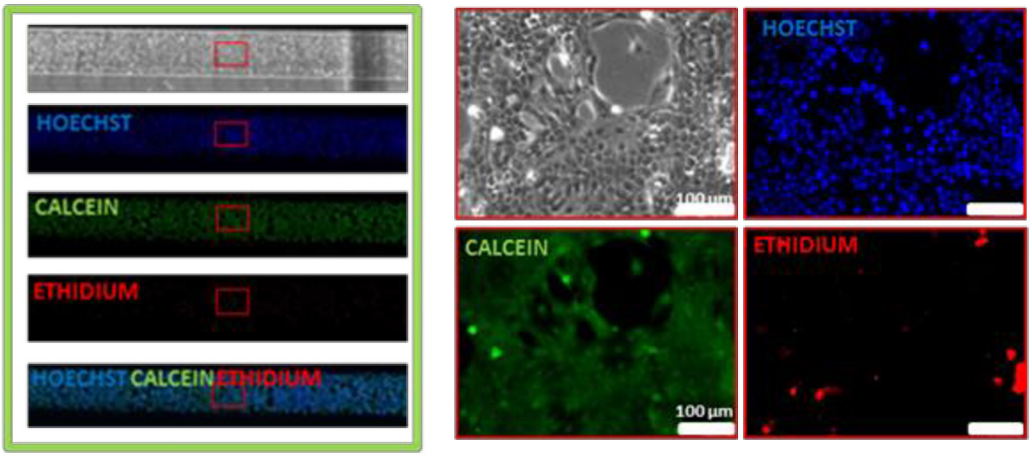

Figure 3.8 LIVE/DEAD assay after 24h of luminescence signal acquisition on HaCaT cells. Green fluorescence identifies living cells , red colour identifies dead ones while Hoechst stain the nuclei.

To ensure the absence of experimental bias due to liming reagent during the real-time luminescence analysis we maintained the cells with or without a glass coverslip on the top of a petri dish in which was insert the microfluidic platform to vary the oxygen amount along the time of the experiment. In this latter case the microfluidic platform was completely enclosed in the case , without any external oxygen access. In addition we collected the medium after 12 hours from the acquisition and tested on different batch of cells analysing again the luminescence signal. In both case was observed the same result (data not shown).

3.2 Real-time analysis of TGF-β pathway activation

We performed a quantitative analysis of the luciferase activity associated to TGF-β stimulation by measuring the luminescence signal in real-time during a period of 24 hours.. The analysis of cell response to sustained TGF-β stimulation results in a transient signal with a peak of intensity after 3.5 hours and a return to the baseline level until 9 hours. We also identified a correlation between different TGF-β1 concentrations and signaling pathway activation resulting in a different peak intensity maintaining the same dynamic profile (figure 3.9). Because luciferase is stable over the time scale of the experiment and luciferin is not a limiting reagent, luciferase activity should continue to increase as long as the signaling pathway is active. The dynamic signaling inactivation after 3.5 hours shows, instead, a desensitization to TGF-β ligand in the extracellular compartment. Our results suggest that upon sustained TGF-β stimulation a negative feedback elicit a desensitization in a range of 3.5 hours. To follow the kinetics of signaling associated to the intracellular dynamic it was used SB431542, the specific inhibitor of TGF-βRI kinases, that blocks the intracellular signaling. Cells was stimulated with TGF-β1 and then added SB431542 at variable times after stimulation. The time point at which the addition of the inhibitor no longer affect the pathway dynamic reflect the time range that is sufficient to induce the transient dynamic in target cells. We observed that by adding TGF-β and SB431542 at the same time resulting in an absence of signaling pathway activation, while adding SB431542 after more than one hour from TGF-β stimulation doesn't affect the pathway dynamic (figure 3.10). These results

suggest that the dynamic is not correlated to sustained stimulation with TGF-β, but is regulated at intracellular level.

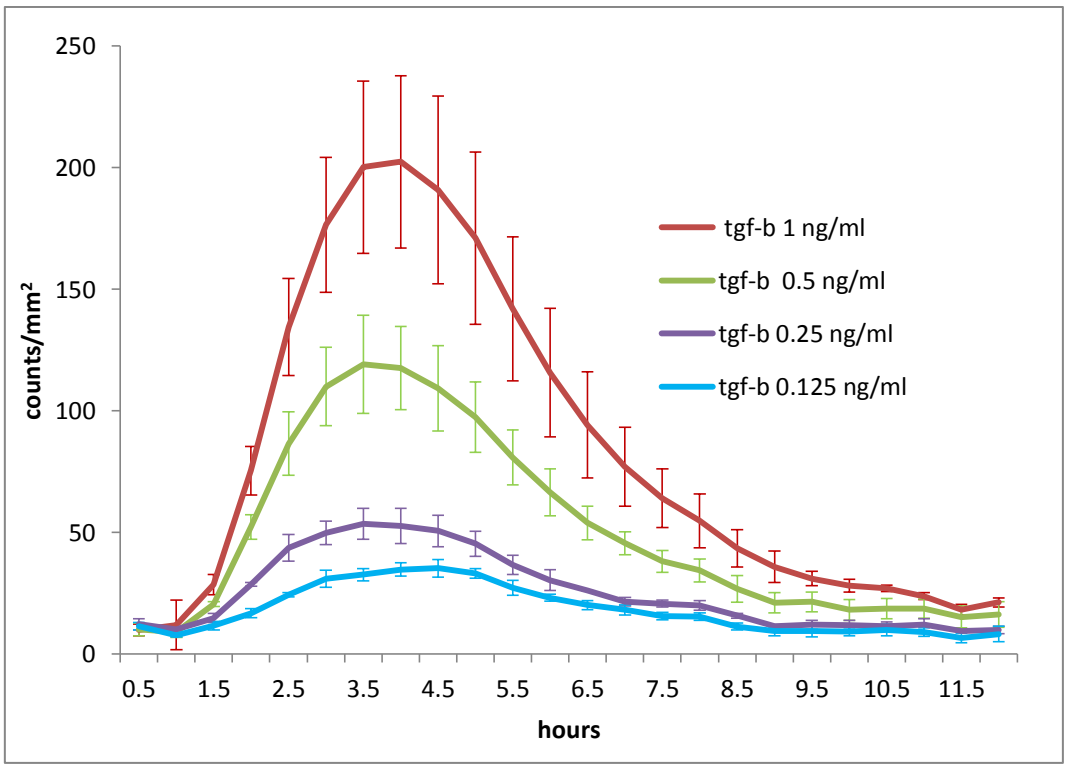

Figure 3.9 Luminescence signal normalized on surface unit, measured on reporter cells during the time following stimulation with TGF-β at different concentration.

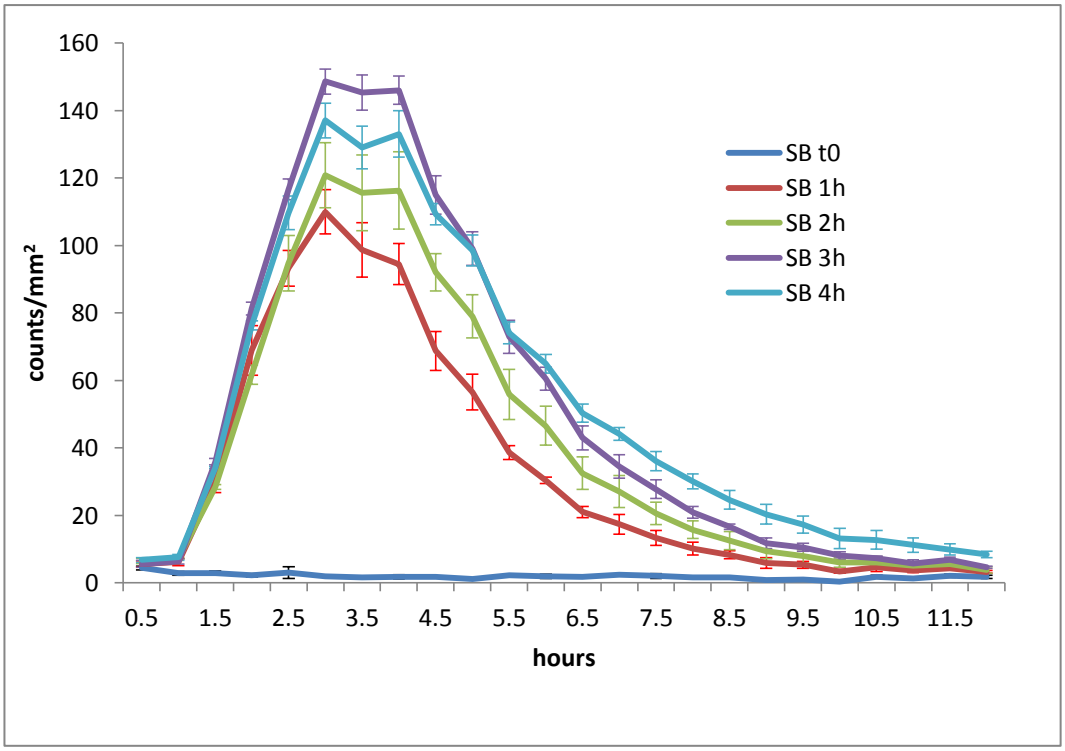

Figure 3.10 Effect of SB431542 inhibitor on TGF-β dynamic depending on time of addiction after TGF-β 1 ng/ml stimulation (from t0 to 4h).

Based on these evidence was also investigated the effect of multiple TGF-β stimulation on pathway dynamic. HaCaT cells was stimulated two times at t0 and after 3 or 7 hours from the first stimulation. Figure 3.11 shows that the second stimulation at 3 hours elicit an increase of signaling with a second peak at 5 hours, while the stimulation at 7 hours has no effect on pathway dynamics (figure 3.12), confirming the desensitization to TGF-β ligand 3.5 hours from the stimulus. In order to quantitatively assess TGF-β signaling to pulsatile stimulation, HaCaT cells was treated with TGF-β for 1 hour, then washed with PBS and incubated with fresh medium containing TGF-β. Data obtained indicate that pulse stimulation every 1 hour result in an increase of peak intensity at 3.5 hours without affecting the duration of pathway activation (figure 3.13).

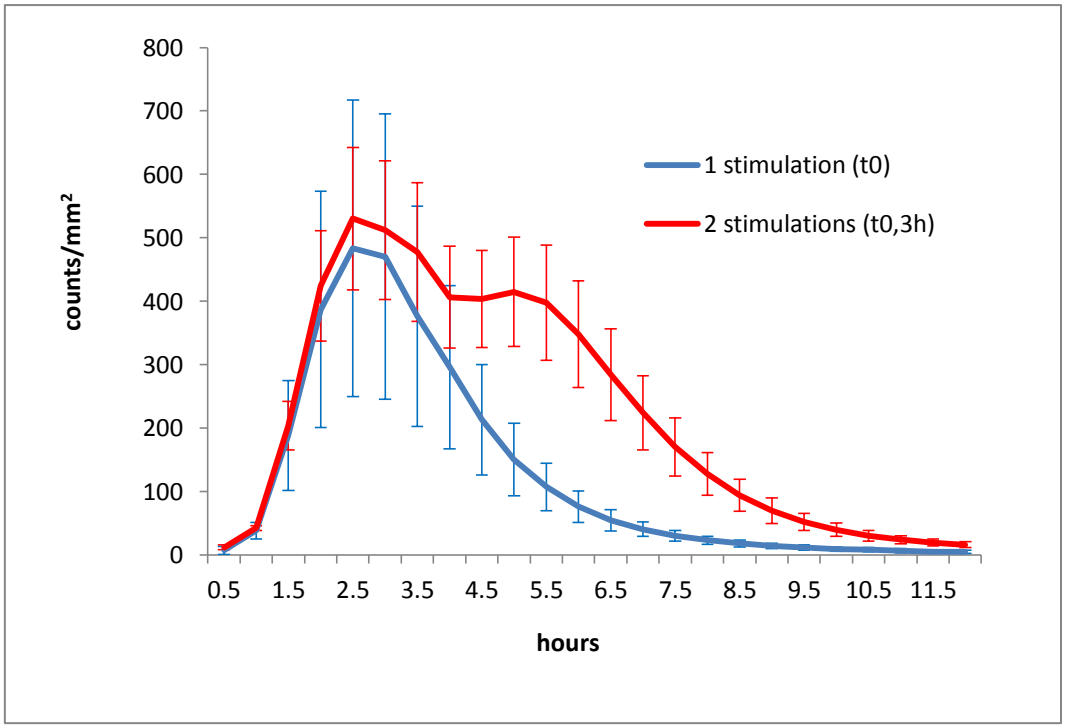

Figure 3.11 Luminescence signal normalized on surface unit, measured during the time following one TGF-β (1 ng/ml) stimulation at t0 (blue line) and two stimulation at t0 and 3h (red line) on reporter cells.

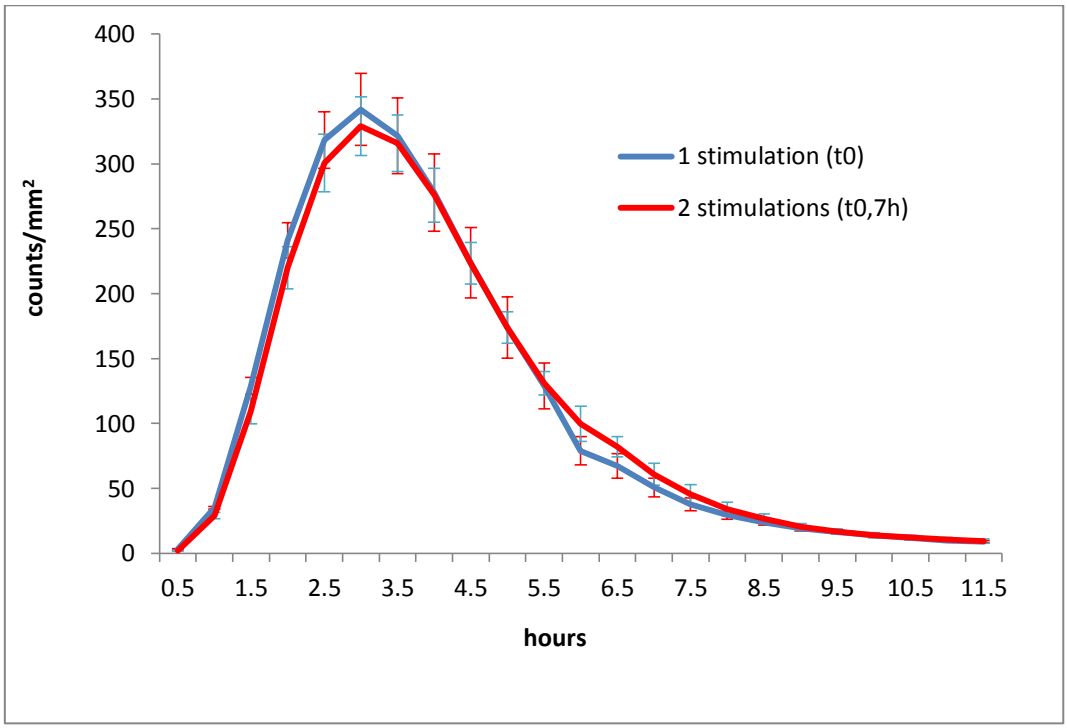

Figure 3.12 Luminescence signal normalized on surface unit measured during the time following one TGF-β (1 ng/ml) stimulation at t0 (blue line) and two stimulation at t0 and 7h (red line) on reporter cells.

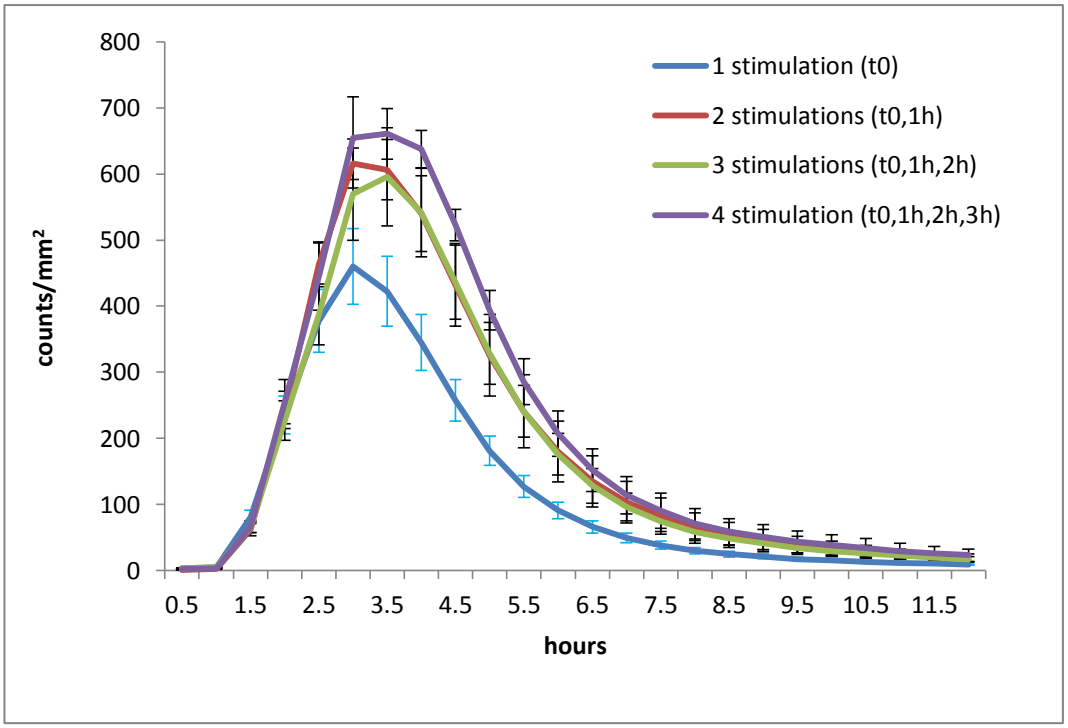

Figure 3.13 TGF-β dynamics following additional TGF-β 1 ng/ml stimulation (from 1 to 4 stimulation) at frequency of one hour.

3.2.1 Transcriptional response to TGF-β reveals a dynamic expression of target genes

In order to follow how the kinetics of the signaling pathway is associated to endogenous target gene expression cells was stimulated with TGF-β and analysed the dynamic of specific target genes by qRT-PCR. It was analysed the gene expression of three specific genes of TGF-β pathway: PAI1, SMAD7 and SNAIL1 , to revealing how specific genes are differentially expressed based on stimulation profile. Sustained stimulation results in a transient response with a peak of expression after 2 hours, returning to baseline after 6 hours. HaCaT cells treated two times, with a second time at 3 hours from the first stimulus showed an increase of PAI1 expression at 6 hours, returning to baseline at 9-12 hours. Surprisingly the dynamic expression of SNAIL1 and SMAD7 is not affected from

the second stimulation (figure 3.14). Is it possible to hypothesize that a balance of expression of target genes and inhibitory SMAD7 regulate both the dynamic and duration of the pathway. Negative feedback following stimulation is a common feature of the signal transduction pathways that would tend to lead to partial adaptation. It was demonstrated that performing pulsatile TGF-β stimulation it is possible to modify gene expression of specific target genes of TGF-β pathway. In particular, was observed that an increase of PAI1expression without a SMAD7 increment results in a modulation of pathway dynamic with a second peak of intensity at 5.5 hours although the total duration of the pathway was unaffected. This result demonstrates how the cells can read TGF-β concentration in the medium modulating the expression of specific genes.

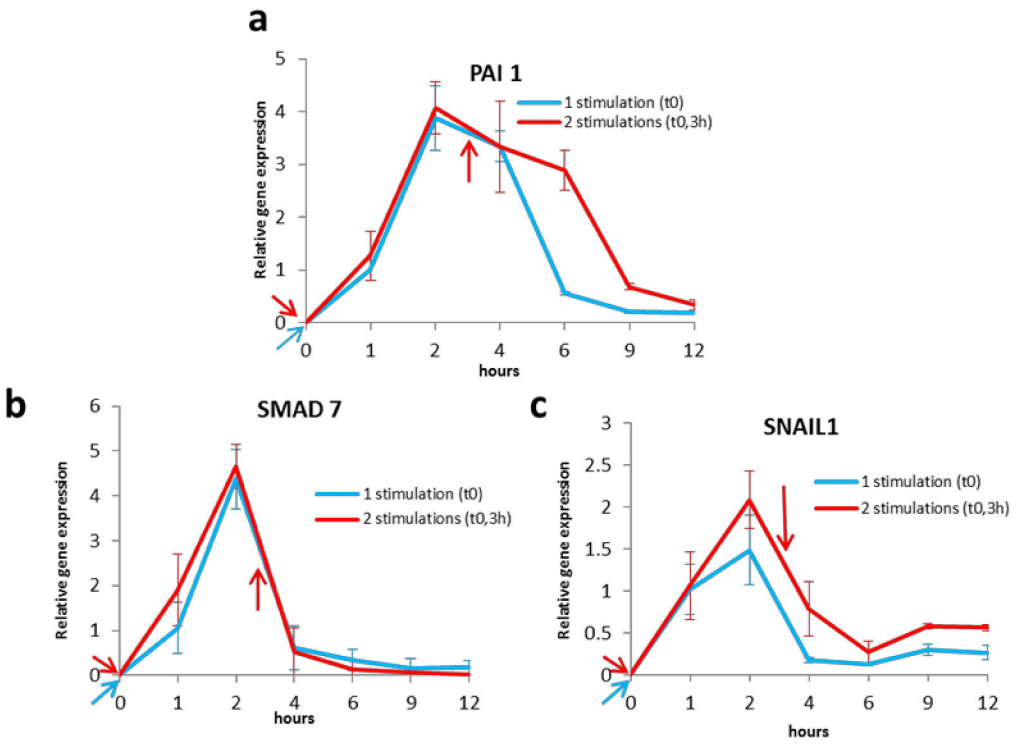

Figure 3.14. Expression of TGF-β target genes by qRT-PCR as a function of time after TGF-β 1ng/ml treatment. (**a**,**b**,**c**) PAI1, SMAD7 and SNAIL1 transcript monitored at different time point after TGF-β stimulation revealing different transient dynamic under continuous stimulation or

performing a second stimulation at 3 hours on HaCaT cells. Data are express as mean of 3 indipendent experiment ± standard deviation.

It is known that TGF-β can trigger different outcomes depending on target cells type, especially in differentiated or pluripotent stem cells. TGF-β activates SMAD transcription factors, then the cell leads target genes expression trough the action of co-factors. Following the dynamic gene expression of PAI1 and SMAD7 in HaCaT and HES-2 cells we investigate how differentiated al stem cells respond to a sustained TGF-β stimulation. PAI1 induction in HES-2 cells have a different dynamic compared to HaCaT, with a peak of expression at 4 hours, and return to baseline in 12 hours. Conversely, SMAD7 expression is quite similar, with a peak at 2 hours, returning to baseline after 6-9 hours (figure 3.15). Balanced expression of the same target genes in different cells could reflect a different functional response. The duration of the signaling response is thought to be an important factor influencing the cell's phenotypic response to TGF-β.

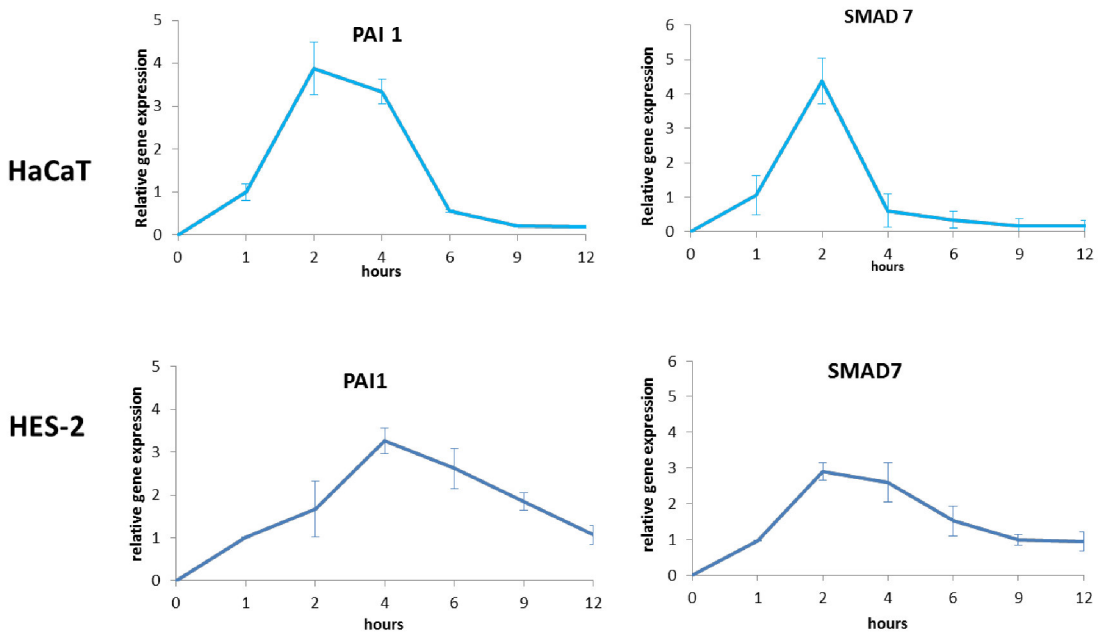

Figure 3.15. Expression of PAI1 and SMAD7 monitored at different time point after TGF-β 1ng/ml treatment in HaCaT and HES-2. Data are express as mean of 3 indipendent experiment ± standard deviation.

3.2.2 Analysis of the role of SMAD7 on pathway dynamic modulation

TGF-β stimulation elicits a transitional gene expression of SMAD7. In order to analyse how the transient transcriptional dynamic reflects protein expression, we also performed immunofluorescence and western blot analysis on HaCaT cells stimulated with TGF-β for 3 or 6 hours. Figure 3.16 shows the expression of the protein at a basal level and an increase of expression due to TGF-β stimulation with an higher level 6 hours after the stimulus.

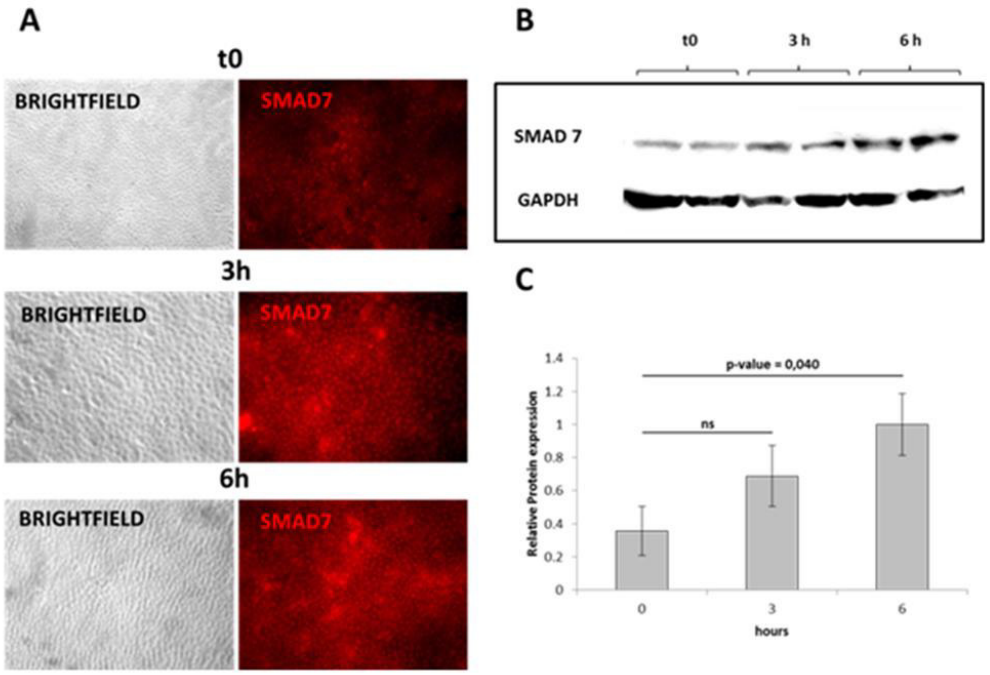

Figure 3.16 SMAD 7 protein expression analysis at different time point after TGF-β 1 ng/ml stimulation (t0,3 hours and 6 hours) using immunofluorescence(A) and Western blot on cell lysate (B). Relative protein quantification was normalized on GAPDH and are express as mean of three different biological replicate ± standard deviation (C).

In order to elucidate the role of SMAD7 on TGF-β negative regulation it was also performed SMAD7 silencing mediated by SMAD7 siRNA. Firstly, was validated

the silencing protocol by western blot (figure 3.17) and the silencing effect on PAI1 expression. Results reported in figure 3.18 shows PAI1 upregulation in a range of 12 hours following TGF-β stimulation, due to SMAD7 downregulation. This data demonstrate that SMAD7 is directly correlated with the mechanism that regulates the transcription of specific target genes of TGF-β pathway.

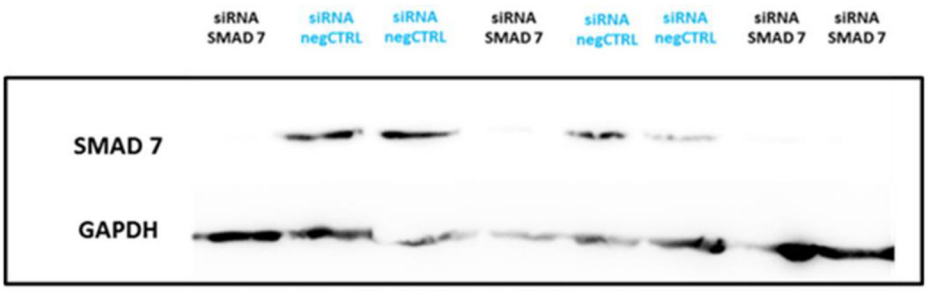

Figure 3.17 Western Blot analysis on HaCaT cells transfected for 72 hours with SMAD7 siRNA and negative control after 4 hours from the TGF-β stimulus.

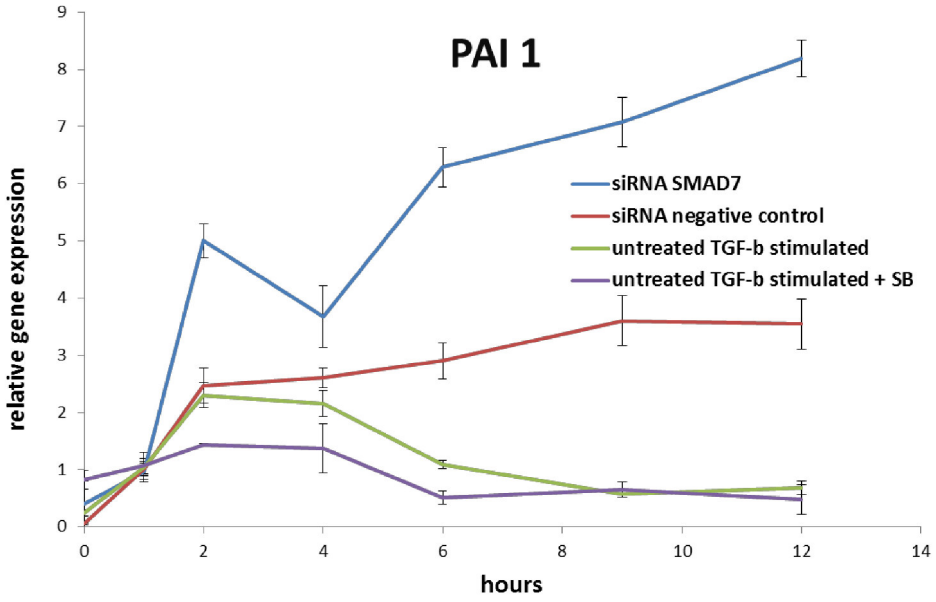

Figure 3.18 Gene expression dynamic of PAI1 following TGF-β 1 ng/ml stimulation in HaCaT cells treated with SMAD7 siRNA and negative controls. Data are express as mean of 3 indipendent experiment ± standard deviation.

CHAPTER IV

Modulation of TGF-β secretion, activation and function by cells of the hepatic microenvironment in a microfluidic system

TGF-β is involved in wound healing processes and also regarded as a central regulator in the development of various liver diseases because it contributes to all the stages of disease progression. Although TGF-β is involved in the maintenance of tissue homeostasis, under both normal and dynamic conditions, alterations in the signaling pathway are often associate to fibrotic state. A range of liver cells are considered to secrete TGF-β ligands and express related receptors, playing a crucial role in the progression of liver disease. In this context it was analysed the TGF-β production mediated by cells of the hepatic microenvironment both in active and inactive form using the microfluidic system.

4.1 Endogenous TGF-β accumulation in a microfluidic system

The intrinsic properties of microfluidics allow the spatiotemporal control of the cell culture microenvironment. Culturing cells in a confined environment gives the possibility to enhance the concentration of secreted soluble ligands in a way impossible to obtain in traditional well system. Using this technology it was

investigated the endogenous TGF-β production mediated by liver cells (hepatocytes, HSCs, macrophages and HepG2). In order to optimize the TGF-β detection system it was used HES-2 cells, which are recognized to produce autocrine TGF-β to promote pluripotency and germ layer specification [1-2]. HES-2 cells are traditionally maintained in culture using medium containing TGF-β: TeSR-E8 (Stemcell Technologies). In order to remove any effect due to the exogenous TGF-β , the culture medium was switched to TeSR-E7 (Stemcell Technologies), with the same composition except TGF-β, 12 hours before to starting to collecting conditioned medium. We analysed the endogenous TGF-β production during 48 hours both in 96 multiwell system (using 150 μl of incubation medium) and in microfluidic system. To assess the total amount of TGF-β in the conditioned medium is needful activate the latent form. Thus, it was tested the conditioned media as it is or heat activated (HA) for 5 minute at 95°C to activate TGF-β in latent form. As positive luminescence control it was used TeSR-E8 medium, while TeSR-E7 and conditioned medium with SB431542 inhibitor were used as negative controls. Figure 4.1 shows that heat activated conditioned medium from HES-2 cultured in microfluidic system induce a significant luminescence signal on HaCaT cells compared to negative controls. On the contrary, conditioned medium obtained in traditional well culture not shown any different signal from the negative controls. Furthermore it was observed that also not thermally treated medium induce luminescence signal, demonstrating that HES-2 produce TGF-β partially in active form.

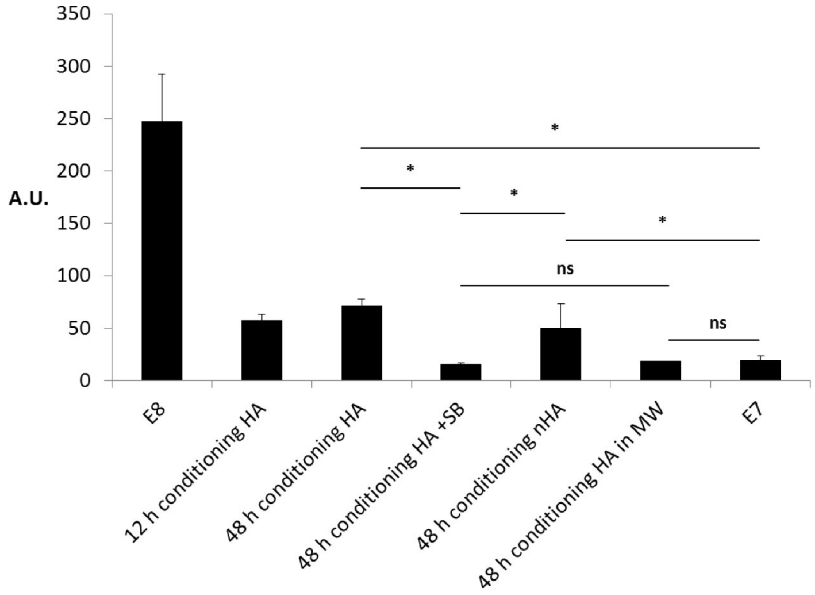

Figure 4.1 luminescence analysis on HaCaT cell lysate after stimulation with HES-2 conditioned media. From left to right are indicate the luminescence values obtained using TeSR-E8 medium, heat activated (HA) medium obtained after 12 and 48 hours from HES-2 cells, the same medium in presence of SB431542 inhibitor, the same medium not heat activated (nHA) or obtained in multiwell system and TeSR-E7 medium. Data are express as mean of 3 indipendent experiment ± standard deviation. Multiple comparisons were performed by one-way ANOVA with Tukey post-test, with $P \leq 0.05$ (*) or $P \leq 0.01$ (**) indicating significance.

The activation of TGF-β signaling pathway was also analysed by monitoring the luminescence production in real-time. Results in figure 4.2 show how conditioned medium both heat activated (total) or not heat activated (active) induce transient TGF-β pathway activation compared to stimulation with exogenous 1 ng/ml TGF-β . As a negative control we used conditioned medium with SB431542.

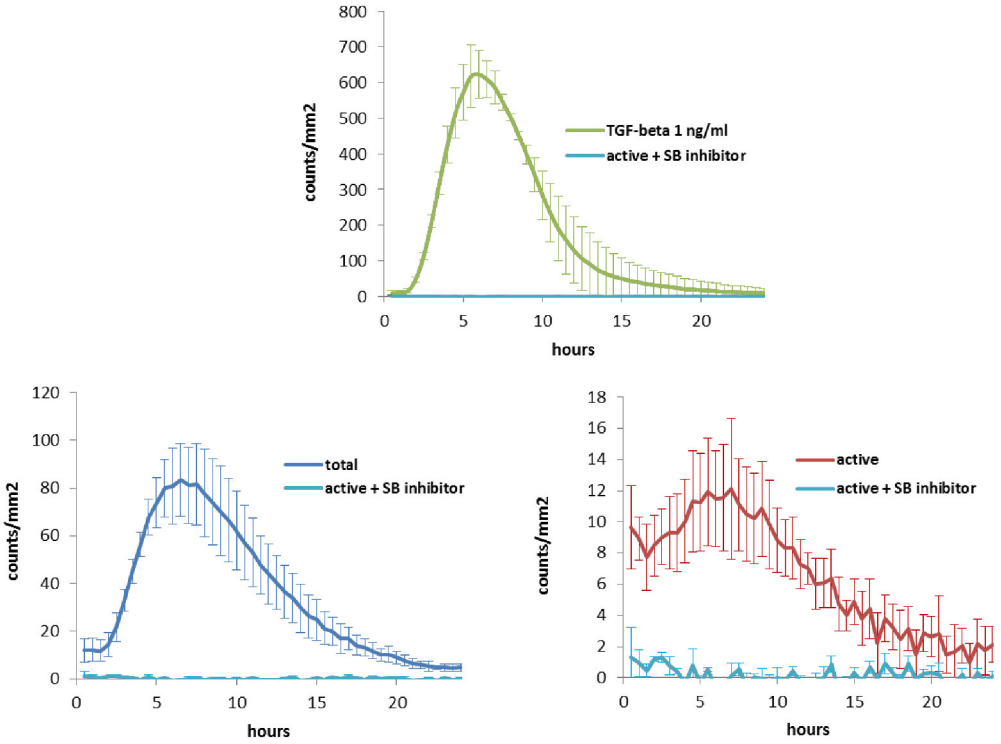

Figure 4.2. TGF-β pathway dynamic activation in HaCaT cells mediated by TGF-β 1ng/ml and conditioned media from HES-2, as it is (active) or heat activated (total) to activate the Latent TGF-β complex and conditioned media added with SB inhibitor as negative control (active + SB inhibitor). Data are express as mean of 3 indipendent experiment ± standard deviation.

4.2 TGF-β production mediated by cells of the of the hepatic microenvironment

Autocrine and paracrine secretion of TGF-β from cells of the hepatic microenvironment contributes to regulate the fibrotic process mediated by hepatic stellate cells [3]. By using microfluidic technology, it was possible to analyse the production of TGF-β in active or inactive form mediated by cells involved in the inflammatory and fibrotic process. It was used HepG2 cell line, commonly used in metabolic study, as *in vitro* model of human hepatocyte , human stellate cells LX-2 and also human macrophages derived from circulating monocyte. TGF-β in

active form was valuated stimulating HaCaT cells with conditioned medium not activated, while total TGF-β was quantified using the same conditioned medium heat activated for 5 minute at 95°C. Figure 4.3 shows that HepG2 cells doesn't secrete TGF-β both in active or in inactive form. Stellate cells indeed, secrete a large amount of TGF-β mostly in inactive form. On the contrary unpolarized macrophages M0, and also polarized macrophages M1 and M2 secrete TGF-β completely in active form. Activation of TGF-β latent complex is a process regulated by many factors like circulating molecules or extracellular matrix component secreted both in autocrine and paracrine manner in different cells. This latter evidence could explain the autocrine TGF-β in active form mediated by macrophages.

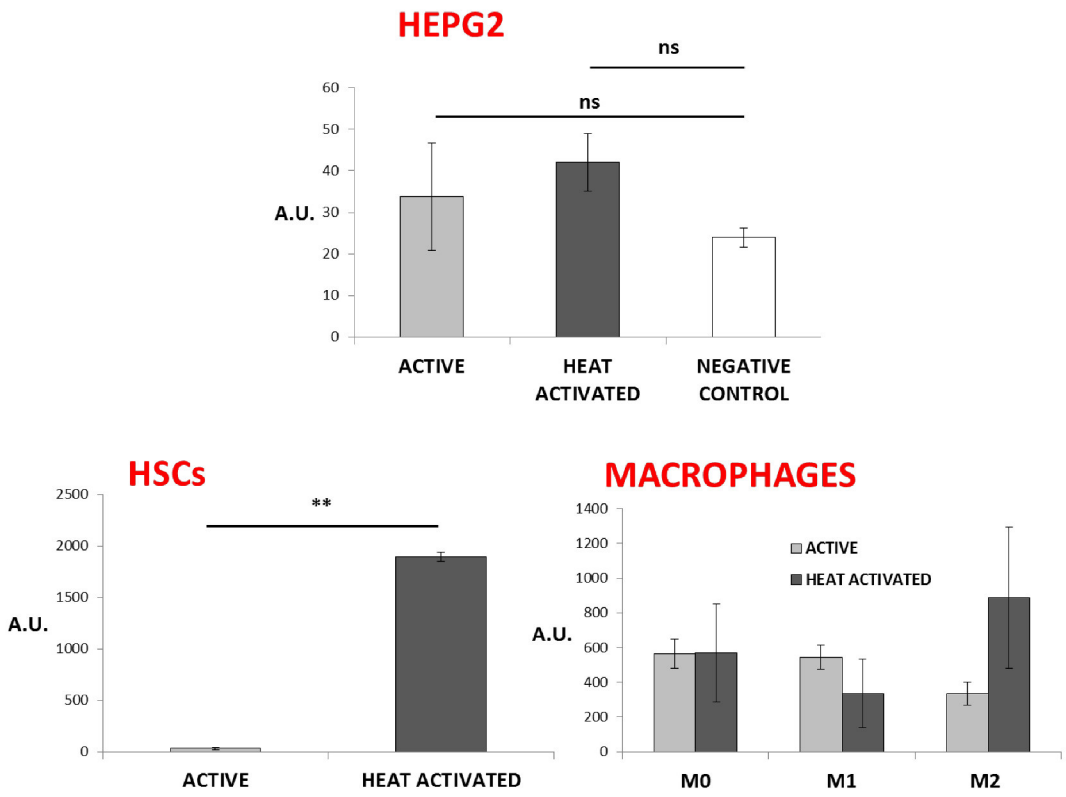

Figure 4.3 Active and total (heat activated) TGF-β production mediated by hepG2, HSCs and different macrophages phenotype M0, M1 and M2 valuated by measuring luminescence production : AU (luminescence Arbitrary Unit) in HaCaT TGF-β reporter cells. Negative control is

represented by conditioned medium with SB431542 inhibitor. Data are express as mean of 3 indipendent experiment ± standard deviation. Single pairwise comparisons were analyzed using Student's t-test with $P \leq 0.05$ (*) or $P \leq 0.01$ (**) indicating significance.

4.3 TGF-β activation mediated by thrombospondin-1 in human stellate cells

Hepatic stellate cells are the mainly responsible cells for the collagen production during the fibrotic process in liver. Collagen production is stimulated by autocrine TGF-β production and also by paracrine stimulation from the cells of the hepatic microenvironment [4]. Cells-secreted TGF-β requires an enzymatic activation process to bind his specific receptor. Thrombospondin-1 is an extracellular matrix glycoprotein expressed at low level in physiologic condition and upregulated in patients affected by hepatic fibrosis. Based on this evidence, we evaluated the effect of thrombospondin-1 (1μg/ml) on TGF-β secretion in HSCs conditioned medium. As experimental control for the activation of TGF-β we also tested the effect of thrombospondin-1 on TGF-β production mediated by HES-2 cells. In figure 4.4 is it possible to appreciate how thrombospondin-1 produce an increment of TGF-β active molecules both in HES-2 and HSCs but is not able to activate the total latent form.

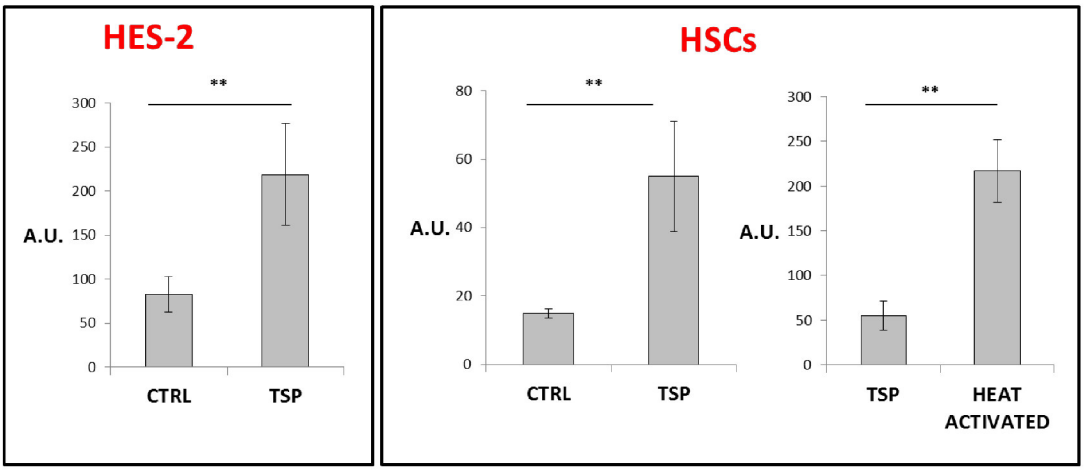

Figure 4.4 Thrombospondin effect on TGF-β latent form activation in HES-2 and human stellate cells (HSCs). CTRL represent conditioned media from cells not treated with thrombospondin and also totat. The total TGF-β was valuated using heat activated conditioned medium. TGF was quantified by measuring luminescence production : AU (luminescence Arbitrary Unit) in HaCaT TGF-β reporter cells. Data are express as mean of 3 indipendent experiment ± standard deviation. Single pairwise comparisons were analyzed using Student's t-test with $P \leq 0.05$ (*) or $P \leq 0.01$ (**) indicating significance.

4.4 Collagen production induction in cells of the hepatic microenvironment mediated by TGF-β

A functional effect of fibrotic process is the TGF-β-mediated extracellular matrix remodelling and collagen production [5]. In this perspective, it was evaluated the effect of TGF-β on collagen production in cells of the hepatic microenvironment . In addition it was evaluated the effect of blocking TGF-β autocrine stimulation using SB431542 inhibitor, the specific inhibitor of TGF-βRI kinases. Collagen production was measured both in HepG2 cell line and HSCs. HepG2 cells were treated for 48 hours or 6 days, while HSCs just for 48h with TGF-β 1 ng/ml. How is shown in figure 4.5 HepG2 cells stimulated for 48 hours have not revealed a significant variation in collagen production, while is it possible to reveal an

increment after 1 week of incubation. Cells treated with SB inhibitor doesn't show any collagen variation. HSCs on the contrary have shown an increment on collagen in 48 hours and also a collagen reduction in presence of SB431542 inhibitor. This latter result reveals a significant effect mediated by autocrine TGF-β stimulation.

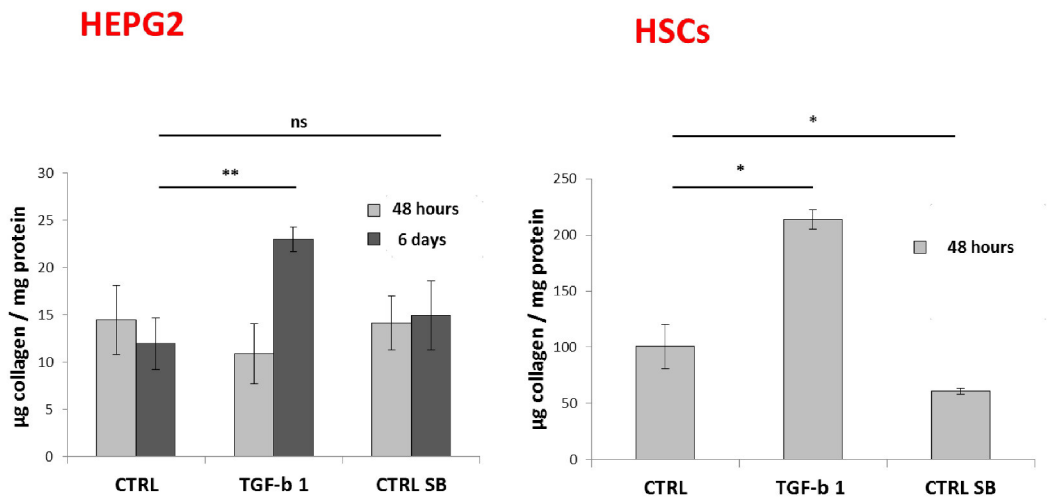

Figure 4.5 TGF-β and SB431542 inhibitor effect on collagen production mediated by HepG2 (at 48 hours and 6 days from the stimulus) and HSCs at 48 hours from the stimulus. Data are express as mean of 3 indipendent experiment ± standard deviation. Single pairwise comparisons were analysed using Student's t-test with $P \leq 0.05$ (*) or $P \leq 0.01$ (**) indicating significance.

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CHAPTER V

Generation of functional hPSCs-derived hepatic cells in a microfluidic system as a model of human hepatocytes

Human primary hepatocyte are the best *in vitro* model to study the hepatic metabolic dysregulation. Metabolic activity is the most important function of hepatocyte *in vitro* and *in vivo*; this function is performed by the complex bio-transforming system, which consist of phase I and II metabolizing enzymes and phase III transporters.

5.1 human primary hepatocyte functional characterization

Metabolic characterization of primary hepatocyte was performed to identify a functional standard for the obtainment of functional hepatic-like cells from hPSCs. Functionality of hPSCs-derived hepatocytes can be assessed through cellular uptake and excretion of indocyanine green. (ICG), which is a unique characteristic of hepatocytes, as well as the capacity of accumulate glycogen, revealed by PAS staining. In figure 4.1A is it possible to appreciate the glycogen accumulation in stained hepatocyte. Another reference assay is the ICG staining ,

that is based on ICG internalization, that confer a green colour to the cells, and metabolism that is visualize by the loss of green colour. In figure 4.1B are show the green hepatocyte immediately after the ICG treatment and 6 hours after the treatment. Hepatic metabolism was also analysed by the induction of cytochrome P450 3A4 mediated by rifampicin stimulation. CYP3A4 expression was measured by qRT-PCR, while enzymatic activity was revealed by CYP3A4 assay using bioluminescence. Rifampicin was added at 3 different concentrations (25, 50 and 100 μM) while DMSO, because of his toxic effect, was used as a positive control for the CYP3A4 induction. Just rifampicin 25 μM induced 30 times increase of CYP3A4 expression comparison to untreated cells. CYP3A4 activity was also demonstrated by the induction of luminescence signal in cells treated with rifampicin. The obtainment of human primary hepatocyte from liver biopsy is nevertheless an intrusive process. Furthermore primary hepatocyte once plated can be maintained in culture only for five days and can't be expanded.

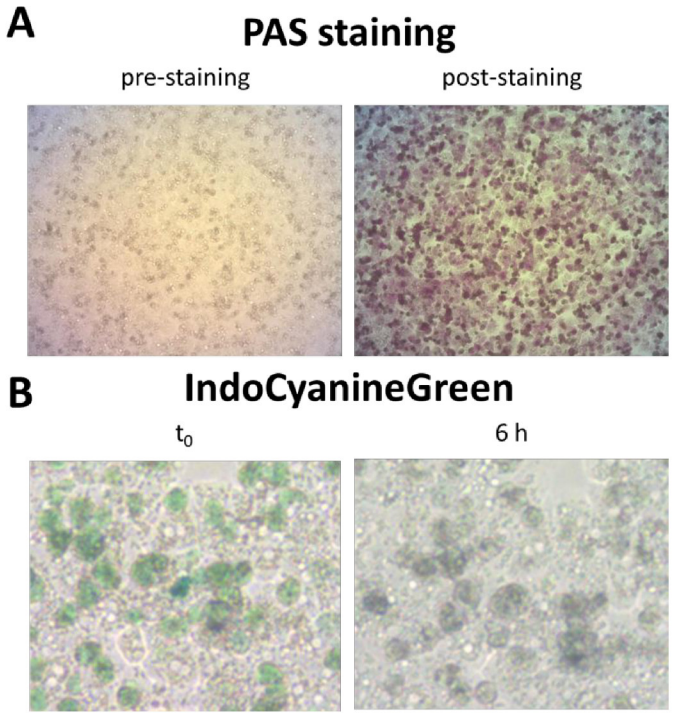

Figure 5.1 Glycogen stained in human hepatocyte treated with Periodic Acid (A). Hepatocyte treated with IndoCyanineGreen and immediately after the treatment and 6 hours later.

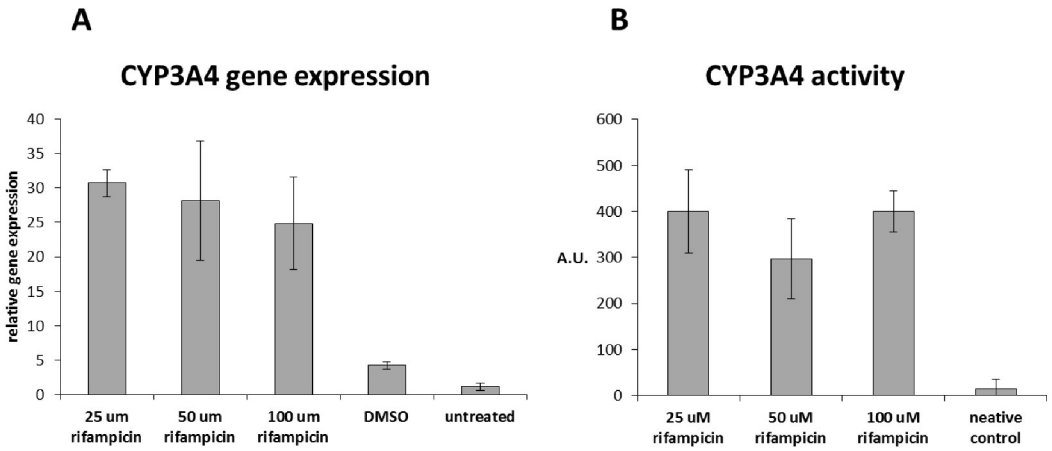

Figure 5.2 CYP3A4 gene expression in human hepatocyte after ryfampicin (25, 50 and 100 μM) stimulation (A). CYP3A4 enzymatic activity in human hepatocyte after ryfampicin stimulation (25, 50 and 100 μM) (B). DMSO was used as a positive control for CYP3A4 induction, while untreated cells were used as a negative control.

5.2 Generation of HES-2 derived hepatic cells

To overcome the requirement of human primary hepatocyte , hepatic-like cells derived from pluripotent stem cells were obtained using a differentiation protocol and characterized as a good model of hepatocyte. Hepatic differentiation protocol was optimized using HES-2 cell line as a positive control for the differentiation of hiPSCs from healty donor or specific patient. This latter issues allow to obtain functional hepatic-like cells for the study of the TGF-β signaling pathway also in hepatic cells derived from fibrotic patients. Cell differentiation was performed using a specific differentiation protocol optimized in our laboratory. The protocol is divided in three phases: endoderm induction, hepatic differentiation and hepatic maturation. The first phase is essential to the differentiation process and takes form the day zero to day 3^{rd} , while hepatic differentiation occurs from the day 4^{th} to day 9^{th} ; finally the complete hepatic maturation occurs until day 18^{th} . Differentiation efficiency was evaluated by the analysis of specific hepatic markers through immunofluorescence. The first step for the differentiation of the hepatic cells is the endoderm specification. High levels of Activin/Nodal, in combination with WNT3a stimulation, induce endoderm formation. The efficiency of endoderm induction was analysed by the expression of specific markers 3 days after the beginning of the differentiation protocol : FOXA2 and SOX17. OCT4 was also analysed as pluripotency marker. In figure 5.2A is possible to appreciate the large expression of both FOXA2 and SOX17. Furthermore is interesting to appreciate the large expression of OCT4, that merge in same cases with SOX17 positive cells, indicating that endoderm marker

expression occurs before the loss of expression of pluripotency marker. Hepatic differentiation occurs starting from day 4th to day 8th , while the complete hepatic maturation can be obtained from the day 9 until the day 17. Hepatic Nuclear Factor α (HNF4a) was analysed as a marker of fully differentiated cells. From the top to the bottom of the figure 5.2 is it possible to appreciate that HNF4a distribution is correlated to cell conformation and distribution. In particular HNF4a is not expressed in high confluency zone that maintain the typical colony conformation of stem cells. This latter issue is probably due to the maintenance of a stem cells niche that produce pluripotency factors. For this reason it was performed a single cells splitting of the stem colony before to starting every single differentiation experiment.

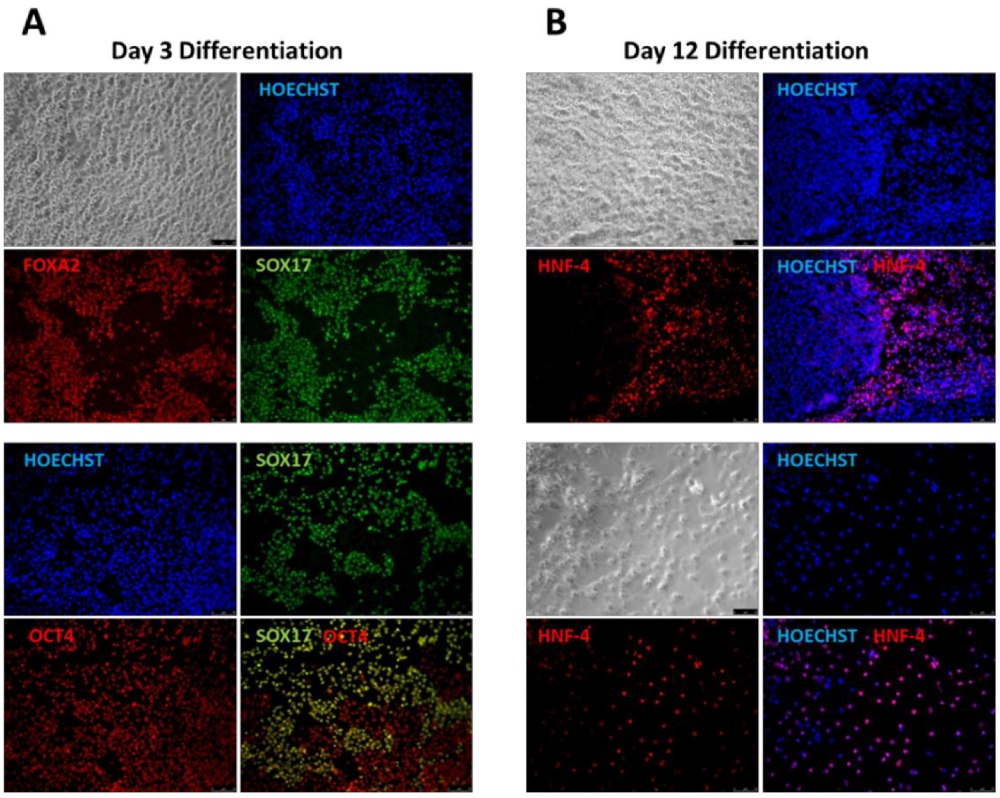

Figure 5.3 immunostaining of HES-2 cells at day 3 and 12 after the beginning of the differentiation protocol. OCT4 was used as a pluripotency marker, SOX17 and FOXA2 stain endoderm positive cells, HNF4a and HOECHST identified respectively hepatic-like cells and nuclei.

5.3 Generation of hiPSCs derived hepatic cells in a microfluidic system

The differentiation protocol optimized on HES-2 cells was extended to hiPSCs, obtained from human fibroblast, in a microfluidic system with the final aim to study TGF-β pathway in hepatic cells derived from specific patients. Endoderm induction was evaluated at day 3 by the expression of FOXA2 and also was analysed the expression of the pluripotency marker OCT4 (figure 5.4A). The immunofluorescence staining was performed on the entire microchannel and identified the expression and distribution of FOX2 in almost all cells. OCT4 was instead expressed only in the high confluency zone, confirming the effect of the stem cell niche in the maintaining of the pluripotency. Hepatic differentiation was evaluated at day 10, identifying HNF4a expression by almost all the cells and the total absence of the pluripotency marker OCT4 confirming the effect of single cells splitting technique increasing the differentiation efficiency (figure 5.4B).

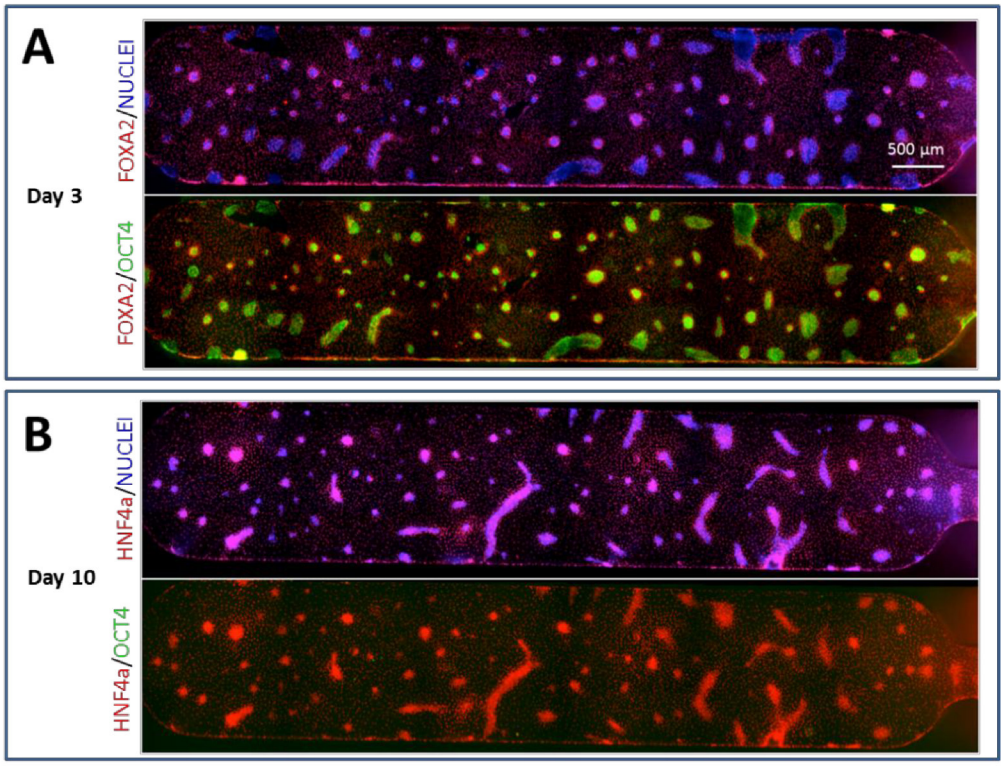

Figure 5.4 Immunostaining of hiPSCs a day third and 10th of hepatic differentiation. FOXA2 stain endoderm positive cells, while OCT4 identified pluripotent cells (A); HNF4a stain differentiated cells (B).

Differentiation protocol is divided in three phases: endoderm induction, hepatic differentiation and hepatic maturation. The last steps occurs from the day 9th to the day 17th and can derive hepatic-like cells that are representative of the primary hepatocyte. Hepatic maturation was characterized at day 20th by the expression of maturation markers. It was analysed the presence of binucleated cells and also the presence of hepatic marker like alphafetoprotein (AFP), albumin, alpha 1 antithrypsin (A1AT) , CYP3A4 and also junction marker like ZO-1, E-cadherin and CK-18 (figure 5.5).

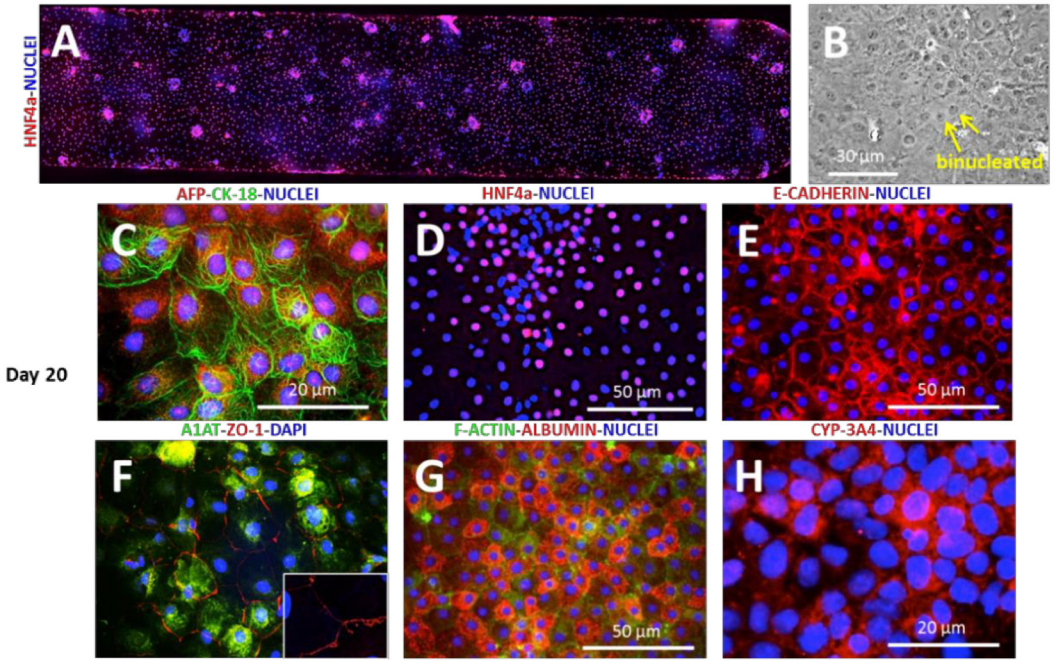

Figure 5.5 Immunostaining of hiPSCs at day 20th of hepatic differentiation protocol. HNF4a expression in almost all the cells (A); binucleated cells observed in phase-contrast microscopy (B); presence of the maturation marker AFP, CK-18 (C); HNF4a (D); E-cadherin (E); A1AT, ZO-1 (F); F-actin, albumin (G) and CYP-3A4 (H).

The expression of endoderm and also hepatic marker during the differentiation protocol was also quantitatively assessed by qRT-PCR. FOXA2 expression increase about 14 times at day 10th and decrease at day 20th confirming the transient induction of endoderm marker. A1AT and AFP was increased during the first 10 days and another high increment was observed at day 20th, demonstrating the large amount of hepatic marker expression at the end of the differentiation protocol (figure 5.6A). The distribution of hepatic markers CYP3A4, A1At and ZO-1 at day 20th and glycogen storage was also compared with human primary hepatocyte, revealing any qualitative difference and also demonstrating the efficiency in the obtainment of hepatic-like cells in the microfluidic system (figure 5.6).

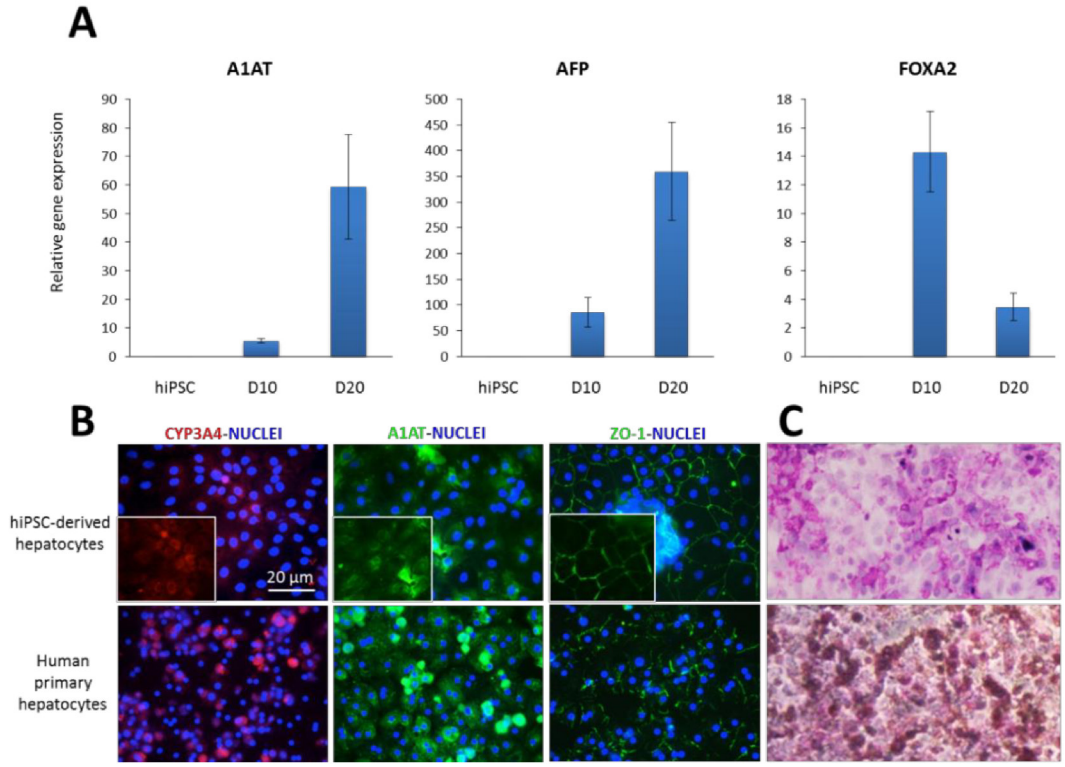

Figure 5.6 relative gene expression of hepatic differentiation markers A1AT, AFP and endoderm marker FOXA2 at day 10th and 20th of the differentiation protocol. Immunostaining of CYP3A4 , A1AT and ZO-1 on hiPSCs derived hepatic-like cells ad day 20th of differentiation and human primary hepatocyte as a reference cells (B); glycogen accumulation analysed through PAS staining in hiPSCs derived hepatic-like cells and human primary hepatocyte.

CHAPTER VI

Modulation of TGF-β secretion mediated by extracellular matrix in a microfluidic platform

TGF-β overexpression is a critical event that drive hepatic fibrosis, inducing modulation of ECM gene expression. This process is well investigated in literature, on the contrary the effect of ECM on cell secretion profile is poor investigated . During the fibrotic process, regulation of production and turnover of ECM components is influenced by TGF-β. Specifically TGF-β have the ability to induce the expression of EMC proteins and stimulate the production of protease inhibitors, that prevent enzymatic breakdown of the ECM [1]. Changing Extracellular Matrix composition could affect the secretion of soluble factors secreted by cells or have an effect on soluble factor retention. In this field we have investigated the effect that the fibrotic matrix could induce on TGF-β production mediated by hepatic cells as profibrotic stimulus.

6.1 Liver acellular healthy and fibrotic matrix characterization

Fibrotic tissues are characterized by the expression of a large amount of collagen isoforms, that contribute to the scar formation. Using acellular matrix derived from healty donors or fibrotic patients it is possible to mimic the effect of the hepatic microenvironment on liver cells. Decellularized liver matrix from healthy donors or fibrotic patients were first of all characterized by the presence and distribution of collagen I and VI. Figure 6.1 shows the fibrillary structure of both collagen I and VI in the healthy matrix. On the contrary acellular matrix from fibrotic patients not express collagen VI, but only collagen I in large amount without any particular fibrillary structure.

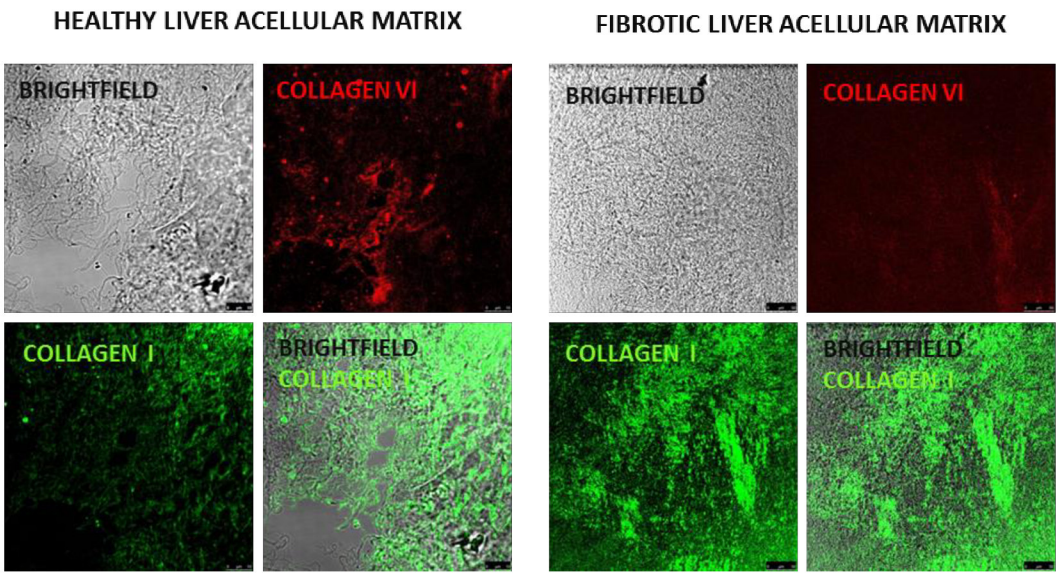

Figure 6.1 Characterization of the presence and distribution of collagen I and VI in decellularized liver matrix from healthy donor or fibrotic patients.

6.2 Hepatic differentiation of hiPSCs on decellularized liver matrix

Specifically designed microfluidic platforms was developed to create microculture chamber in which hiPSCs derived hepatic-like cells could differentiate specifically on healthy or fibrotic extracellular matrix (figure 6.2). Decellularized matrix was frozen in liquid nitrogen, cryosectioned at 40 µm slice and deposed on a polylysine-treated glass surface. A PDMS ring was fabricated to cover all the matrix surface (1.5 cm^2) and create an incubation chamber of 40 µl of volume. An inlet on the top of the microfluidic chamber allows to inject the cells inside the chamber. Exchanging the medium is possible by adding drop of medium on the top of the inlet and drawing the same amount of medium from the outlet.

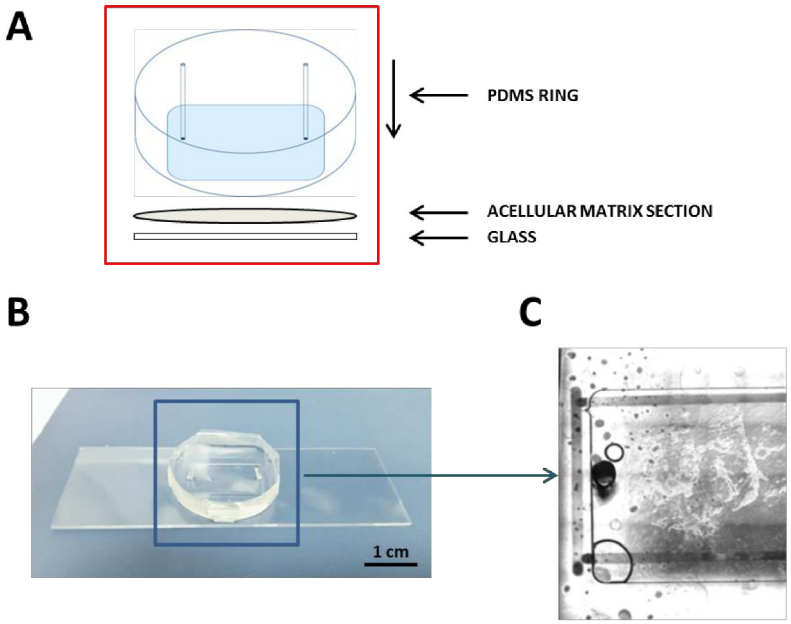

Figure 6.2 Schematic representation of the acellular matrix integration in the microfluidic platform (A); Picture of the microfluidic platform obtained (B); and brightfield microscopy image of the acellular matrix inside the microfluidic platform (C).

Hepatic differentiation protocol was optimized to obtain hepatic-like cell both on healthy and fibrotic acellular matrix. Differentiation protocol was first conducted in 12MW plate with 0.5 % of MRF coating for 10 days. Then the cells were splitted and seeded inside the microfluidic platform. The hepatic maturation protocol was then performed for other 12 days. The efficiency of differentiation process was evaluated both on cells derived on healthy and fibrotic matrix by the expression of HNF4a. How is shown in figure 6.3, differentiating cell on acellular matrix allow to obtain an high differentiation efficiency both on healthy and fibrotic matrix.

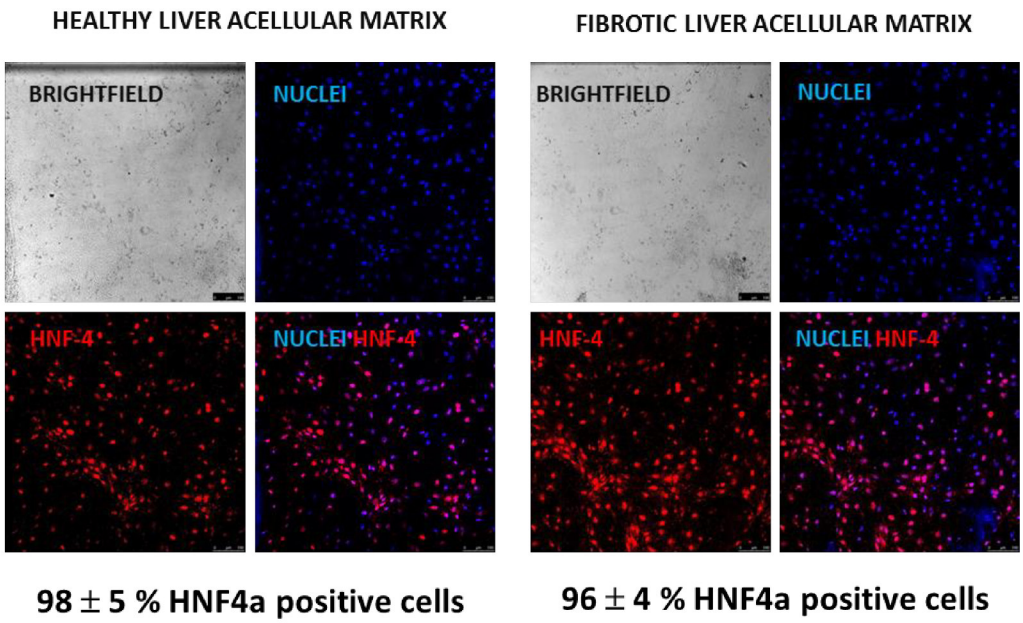

Figure 6.3 HNF-4a differentiation marker expression on hiPSCs derived hepatic-like cells on decellularized liver matrix from healthy donor or fibrotic patient at the end of the hepatic differentiation protocol. Data are express as mean of 3 indipendent experiment ± standard deviation.

6.3 TGF-β secretion modulation by extracellular matrix in hiPSCs derived hepatic-like cells

Extracellular matrix remodelling during fibrotic process is stimulated by the cells of the hepatic microenvironment in response to profibrotic stimuli. The cross-talk between hepatocyte and stellate cells is essential to regulate their activation and perpetuation of fibrotic process. TGF-β is secreted as a profibrotic molecule from the hepatocyte and could have an effect on the activation of HSCs. To analyse if the extracellular matrix can also regulate the expression of profibrotic factors by the hepatic cells we collected the conditioned media obtained from hiPSCs derived hepatic-like cells cultured on the acellular hepatic healthy matrix or the fibrotic one. hiPSCs was differentiated for 22 days collecting the conditioned culture media from the day 20th to 22th. As control we used conditioned media from hepatic-like cells traditionally differentiated on MRF 0.5% surface. The total TGF-β in the conditioned media was expressed as total TGF-β on TGF-β produced by the control sample ratio. As shown in figure 6.4 conditioned medium from cells cultured on fibrotic matrix contain a lower amount of TGF-β in comparison with control and also with cell cultured on healthy matrix. Nevertheless the results were obtained from a single biological replicate and have to be improved by performing further experiments. This result could be related to a negative effect on TGF-β production mediated by fibrotic matrix as a negative feedback to downregulate the fibrotic process. Another way to explain this effect could be related to the properties of the fibrotic matrix to retain TGF-β secreted from hepatic cells, in such a way TGF-β was not bioavailable to activate TGF-β in reporter cells.

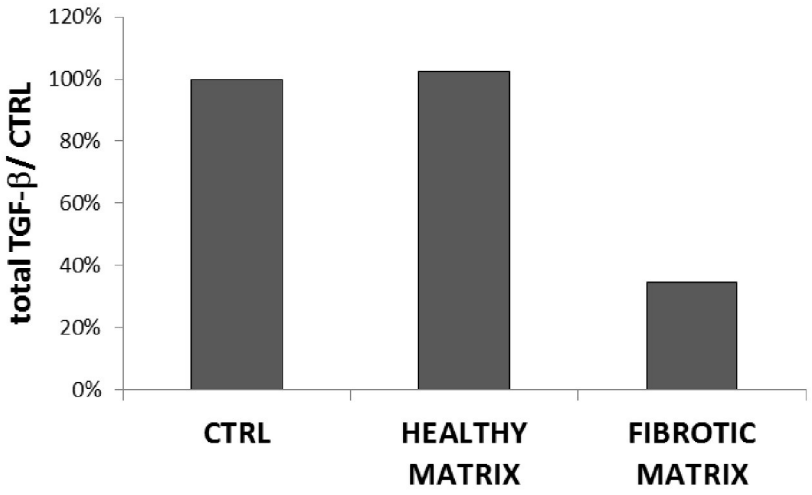

Figure 6.4 Analysis of TGF-β secreted from hiPSCs derived hepatic-like cells differentiated on decellularized extracellular matrix derived from healty donor or fibrotic patient expressed as total TGF-β on TGF-β produced by the control sample ratio.

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Discussion and Conclusion

The main goal of this work concerned the study of TGF-β signaling. TGF-β is one of the most important molecules involved in collagen production during fibrotic process in many organs and tissues. In particular, the fibrotic process in liver is mediated by stellate cells, through extracellular matrix remodelling and collagen production to repair the damaged tissue. The overexpression of collagen results in a non-functional process in which the damaged hepatocyte are substituted with fibrotic tissue. Anti-fibrotic therapy aims to downregulate collagen production blocking TGF-β signaling. Nevertheless blocking TGF-β signaling with low specificity may lead to unknown adverse reactions. In this field is essential to understand the mechanism of pathway activation and TGF-β latent form activation for the study of new anti-fibrotic therapy. Using a microfluidic system it was possible to perform a real-time analysis of TGF-β pathway activation and identify a correlation between stimulation frequency and pathway dynamic. It was also valuated the expression of specific target genes in response to frequency stimulation. Traditionally used technique to study TGF-β signaling comprise Western Blot, genic expression assay and luminescence assay on cell lysate. Measuring luminescence production during the time allow the possibility to study pathway activation dynamic, identifying a transient response to TGF-β. Several intracellular mechanism regulate TGF-β signaling. TGF-β binding to its receptor

induce R-SMADs phosphorylation , but is the co-SMAD that regulate genic expression in the nucleus. SMAD 7 inhibit the signaling by a negative feedback mechanism. PAI1 is one of the most important target gene that revealed a peak at 2 hours after sustained TGF-β stimulation and return to basal expression in 6 hours. SMAD7 expression was revealed also transient and indipendent on stimulation frequency, with a peak at 2 hours from the stimulation that return to basal level in a range of four hours. At the same time protein analysis revealed an increase of SMAD7 expression after TGF-β stimulation with a peak at 6 hours. SMAD7 silencing also showed an effect on PAI1 temporal expression that increase in a range of 12 hours. Overall these data suggest that SMAD7 inhibition produce a substantial modification of TGF-β dynamic with a prolonged temporal activation with an important effect in promoting fibrotic process. Taking together, these data contribute to understanding how cells can decode the concentration of extracellular TGF-β and transduce with a different intracellular mechanism. The TGF-β pathways elicits a transient signaling in response to continuous or pulsatile stimulation, but with a different dynamic profile. In this work it was developed a novel microfluidic approach to study TGF-β signaling in a real-time manner from the binding of ligand to the receptor, at membrane level, to the transcription of target genes.

A second part of this work was focused on the analysis of TGF-β production, as a latent complex or active molecule mediated by cells of the hepatic microenvironment. In particular was analysed the production mediated by human stellate cells, human macrophages derived from circulating monocyte and hiPSCs derived hepatic-like cells. The microfluidic platform allowed to concentrate secreted soluble molecules to a level impossible to obtain in traditional well and is

essential to identify the autocrine TGF-β production. HSCs secrete large amount of TGF-β mainly in inactive form , hepatocyte produce a low amount of TGF-β. Macrophages on the contrary produce a large amount of TGF-β completely in active form. This data explains the autocrine production of TGF-β in active form. Microfluidic system was also used to evaluate the effect of thrombospondin-1 on TGF-β latent form activation. Thrombospondin is an important target because is produced at low concentration from cells of the hepatic microenvironment and its concentration is increased in the serum of fibrotic patients. Sartans , angiotensin II receptor antagonist block fibrosis decreasing thrombospondin production induced by angiotensin II. Using the microfluidic system it was revealed that thrombospondin-1 increase TGF-β active form produced by stellate cells, acting as a profibrotic factor. However the activation mediated by thrombospondin is only partial, demonstrating that activation of TGF-β latent form is a complex and multifactorial system. Functional effect of TGF-β on collagen production was evaluated in HepG2 and HSCs. TGF-β increase the collagen production in HepG2 cells after 1 week of culture and also in HSCs after 48 hours of TGF-β stimulation. Moreover, not treated HSCs maintained in culture with SB431542 inhibitor showed a lower level of collagen production, demonstrating that blocking autocrine stimulation results in a decrease of collagen production.

A third part of the work was focused on the development of a hiPSCs derived hepatic-like cells model. The obtainment of human primary hepatocyte for the study of metabolic activity and the production of endogenous factors is complicated by the need of biopsy and the impossibility of performing cell expansion. In this context is extremely difficult and expensive performing experiments on human primary hepatocyte. Furthermore the immortalized hepatic

cell lines not represent a good model for metabolic studies. Differentiating hepatic-like cells in a microfluidic system provide a unique technology for creating a microenvironment in which hepatic-like cells concentrate soluble secreted factors for autocrine stimulation. The differentiation protocol was also optimized for the obtainment of hepatic cells on acellular matrix of healthy donor or fibrotic patient. It was demonstrated that fibrotic matrix contains a large amount of collagen I compared to the healthy one. This model has allowed to perform preliminary experiment to evaluate the effect of the extracellular matrix on autocrine TGF-β production mediated by hepatocyte. Hepatic differentiation efficiency on acellular matrix was evaluated trough HNF4a expression and resulted higher than 90% , demonstrating the high potentially of extracellular matrix in improving the differentiation process. Hepatic-like cells derived showed TGF-β production. The extracellular matrix showed also an effect in modulating TGF-β production in conditioned medium. Fibrotic matrix seems to downregulate TGF-β production in conditioned medium as negative feedback process mediated by fibrotic microenvironment or a retention effect on TGF-β produced, mediated by extracellular matrix.

Concluding, this research on microfluidic technology for the analysis of TGF-β signaling provide new results in understanding the complexity of pathway dynamic and also for the study of the ECM role in fibrotic process. These preliminary results open new prospective for future investigations on potential role of ECM in regulating fibrotic process and the development of new therapeutic target for the treatment of fibrotic diseases.