MICROFLOW INDUCED MECHANOTRANSDUCTION IN HACAT CELLS: A MECHANISTIC STUDY

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CERTIFICATE

This is to certify that research project report entitled "**Microflow induced mechanotransduction in HaCaT cells: A mechanistic study**" submitted by **Tarun Agarwal**, in partial fulfillment of the requirements for the award of the Degree of Master of Technology in Biotechnology and Medical Engineering with specialization in Biotechnology at National Institute of Technology Rourkela is an authentic work carried out by him under my supervision and guidance.

To the best of my knowledge, the matter embodied in the thesis has not been submitted to any other University/ Institute for the award of any Degree or Diploma.

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(**Tarun Agarwal**)

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ABSTRACT

In recent years, fluid flow has been recognized as an important mechanical morphoregulator that governs cell fate *in-vitro* and *in-vivo*. Mechanisms underlying such cellular responses include flow induced shear stress, hydrodynamic pressure or formation of real time morphogen gradient inside the tissue. The epidermal keratinocytes are known to elict mechanosensitive response when exposed to cyclic strain and mechanical stretching; however influence of fluid flow induced shear stress on these cells still remains unexplored. In this regard, we used biomicrofluidics approach to evaluate to influence of flow induced shear stress on cytoskeletal reorganization of HaCaT cells (human keratinocytes) and to understand the mechanism underlying it. The study was carried out in shear stress range of 0.06 dyne/cm^2 to 6.0 dyne/cm^2. The study showed that a low shear stress of 0.06 dyne/cm^2 caused the cellular spreading while the higher shear stress (6 dyne/cm^2) resulted in cytoskeletal damage. It is important to mention that the shear stress of 0.06 dyne/cm^2 elicited the cellular response via ERK1/2 > GSK3β > β catenin pathway. β catenin induces the transcription of proliferative gene, which was confirmed by the expression and nuclear localization of PCNA, proliferation marker. The scanning electron microscopic images of the confluent cell layer exposed to shear stress showed better integrity in comparison to the static control. In reality, though the skin keratinocytes are never exposed to the fluid flow, but these results propose a possible application of fluid flow induced shear stress in the cell sheet engineering *in vitro*.

Keywords

Fluid flow, Shear stress, Epidermal keratinocytes, Cytoskeletal Reorganization, ERK1/2.

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CHAPTER 1

INTRODUCTION AND REVIEW OF LITERATURE

1.1 Mechanotransduction: A brief overview

In the last few decades, the field of mechanobiology has gained prime attention due to its physiological and clinical relevance in nearly every tissue in the body. The researches focused in this domain have tried to address – "how the cells sense the mechanical stimulus and transmit them downstream into a cascade of biochemical signals, resulting into specific physiological response" [1-3]. Another area of in depth research in this field includes the estimation of clinical relevance of these mechanical stresses in various pathobiological states. Various research groups have tried to mimic the *in vitro* natural conditions by fluid shear stress (FSS), cyclic strain, substrate stretch, gravity stress, vibration and shockwaves [3]. In response to these mechanical stimuli, the cells transmit the forces in the intracellular compartment either through cytoskeletal elements or by an excitable molecular entity such as ion channel, membrane receptor or protein spontaneously linked to intracellular signal transduction [4]. With the emerging importance of studying cellular dynamics under mechanical stresses in different physiological processes and pathological conditions, these have resulted into an altogether separate field called Mechanotransduction [2-3].

1.2 Application of mechanotransduction in cellular engineering

The mechanotransduction involves a complex interaction between different cellular components. The increasing evidences suggest the crucial role of cellular components such as integrins, focal adhesion complexes, cytoskeleton, ion channels, cadherins etc. in modulating the cellular behavior [5]. The transmembrane integrins which assist in cellular adhesion and binding with the extracellular matrix is associated with a various kinases such as focal adhesion kinases and associated adaptor proteins including paxillin, vinculin, talin etc. The integrin associated kinases trigger many of the downstream targets including MAP kinases and PI3P/Akt proteins which are further determine the cellular physiological aspects

including its adhesion, proliferation, migration, differentiation and apoptosis [6-7]. The associated adaptor proteins play an important role in linking the cytoskeleton with that of integrins, thus determining cellular motility and proliferation [8]. In addition, the mechanical stimuli such as mechanical stretch, cyclic stress and fluid flow induced shear stress activates the stress sensitive ion channels, which controls calcium influx/efflux and influence the cytoskeletal remodeling and intracellular signaling [9-10]. It is important also important to mention that many of the growth factor and G-protein couples receptor pathways also regulate the cellular response under stress conditions [7], in a ligand-independent manner [11-12]. In this regard, many of the research groups have suggested a crucial role of these mechanical forces in determining the ultimate cellular fate. Shih *et al* reported that mesenchymal stem cells (MSC) showed greater differentiation on collagen type I coated polyacrylamide hydrogel with Young's modulus of 42.1±3.2kPa in comparision to substrate with Young's modulus of 7.0±1.2kPa [13]. Nikukar *et al* demonstrated the use of nanoscale sinusoidal mechanotransductive protocols (10-14 nm displacements at 1 kHz frequency, promoted mesenchymal stem cells to osteoblastogenesis [14]. The matrix stiffness has a crucial role in determining the phenotype and differentiation of mesenchymal stem cells. Many research groups have reported the differentiation of MSC to neural, myogenic and osteogenic when cultured on substrate with elastic modulus in range of 0.1-1kPa, 8-17kPa and 34kPa respectively [15]. Vittorio *et al* has demonstrated that PL-MSCs demonstrated a varied response when subjected to shear stress and substrate of variable rigidity. The PL-MSC when exposed to shear stress in the range of 6–12 dyne/cm2 for 24 h showed increased expression of endothelial markers. Also, when the MSCs when cultured on softer matrices (3kPa stiffness) expressed Flk-1 endothelial marker, while more rigid substrates (>8 kPa) led only to a small percentage (20%) of cells positive for this marker [16]. Also, the hMSC's

developed aligned stress fibres along with increased expression of neurogenic and myogenic differentiation markers when cultured on nanogratings with 250 nm width on PDMS [17].

1.3 Flow induced shear stress mediated mechanotransduction

Fluid flow induced shear stress has been recently recognized as a prime mechanical morphoregulator. The investigations focused in this field have tried to explore the patho-physiological significance of shear stress. It has been already demonstrated that wall shear stress affect many of the cellular processes including cell proliferation, cytoskeletal and nuclear remodelling, adhesion and migration [18]. Such kind of flow plays a crucial role early development of embryo, cell recruitment and homing during tissue repair and regeneration, interstitial protein transport. It is important to mention that under physiological conditions the endothelial cells are exposed to blood flow while, the osteoblasts are exposed to interstitial fluid flow. These fluid flow have been reported that exert a shear stress ranging from 10-20 dynes/cm^2 (endothelial cells) and 7-24 dynes/cm^2 (osteoblasts) respectively, which inturn modulated the overall behavior the cells as well as the corresponding tissue. Clinically, the shear stress finds its relevance in inflammation, tumorogenesis and cancer metastasis [19-20]. Many of the research groups have demonstrated that the fluid flow induced shear stress affects cancer adhesion [21-22], migration [23], invasive capacity [24-25], morphology [25-26] and viability [27].

These fluid flow induced shear stress is transduced into biochemical signals through various mechanoreceptors which forms the intrinsic component of the cell itself. The most potential candidate mechanoreceptors include cell adhesion molecules like Integrins, Cadherins; glycocalyx, shear sensitive or stress activated ion channels, autocrine receptors, some are organellar component of cells like primary cilia and the nuclear membrane proteins or DNA

itself which might undergo conformational changes under influence of fluid flow, thereby affecting the overall gene expression [28].

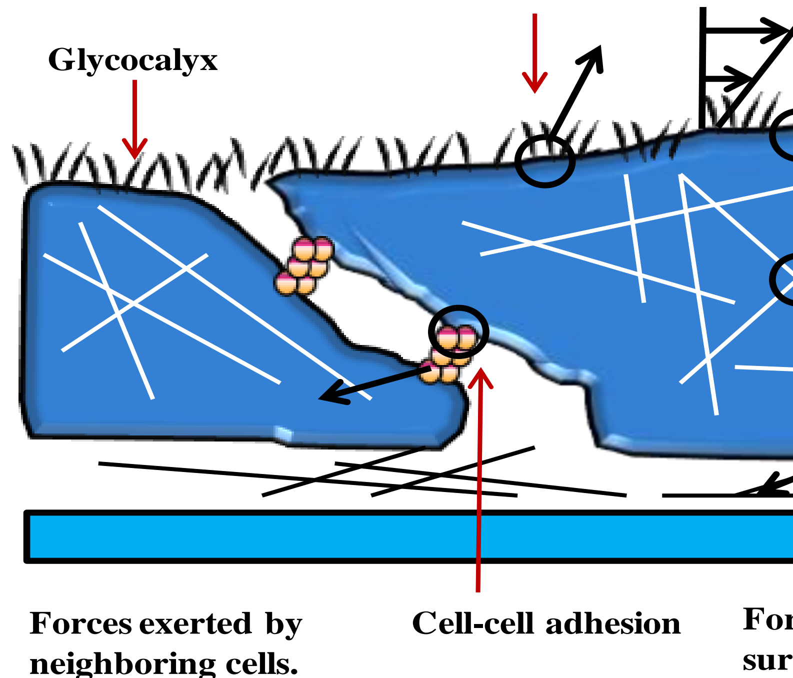


Fig. 1 Fluid flow induced shear stress mediated mechanotransduction and its corresponding mechanotransducers

1.4 Microfluidic system: A smart tool for analysis of influence of flow on cell physiology

Microfluidics deals with liquid of sub-nanoliter volumes through purposefully designed microchannels with at least one of the dimension in micrometer order [29]. The microfluidic system have a number of advantages over the conventional macro-systems including requirement of low the volume of liquid analytes for the assay, providing high throughput, reduced reaction time and high sensitivity due to surface area to volume ratio [30]. The introduction of the microfluidics had been a revolutionary step in the field of molecular biology, biochemistry and bioengineering. The applicability and importance of the microfluidics in these field made it a separate domain termed as Biomicrofluidics [31]. In

addition, the microfluidics has also gained a great importance in the field of cell biology due to its microconfinement which mimics the *in vivo* conditions. The biological cells are dimensionally in the order of micrometers and the microfluidics provides a better platform to analyse cells individually [32]. In some cases, on the basis of specific requirement, even different parts of a single cell can be physically or chemically manipulated though microfluidics [33]. Such functionality allows the use of the microfluidics in fundamental cell biology and associated medical diagnosis [34]. The differential applicability of microfluidics has been in deciphering various intracellular signal transduction pathways under the static and shear stressed conditions of a single cell or several neighboring cells (i.e. chemical communication between different cells or different parts of a single cell) [35]. The microfluidics based system biology has allowed the isolation and analysis of a single cell at a time, which has emerged out to be a distinctive approach to study infected cells *in vitro* [36]. Relevantly, on the basis of deformability, microfluidics based systems have been used for the detection of some of the lethal diseases such as cancer, malaria, AIDS and SARS have been proposed [37]. Again, with pertinence to the problem of distinctive survival advantage of cancer cells, microfluidic systems provide *in vivo* mimic to the micron-sized physiological circulatory-renal flow systems and pores within tissue matrices, thus providing a platform for *in vitro* simulation of *in vivo* cellular behavior [35, 38]. The undeniable similitude between microchannel systems and blood vessels has encouraged researchers to an extent of adopting the technology of microfluidics in the domain of artificial tissue engineering. Moreover, since the illustration of mechanical influence on cell physiology, the fluid shear stress has been most emphatically utilized in the study of mechanotransduction, as the representative stress imparting mechanism. Within microconfinement, due to the dimensional commensurability, it is relatively easy to attain approximately accurate physiological conditions through precise regulation of incipient flow driving mechanisms such as pressure drop [38]. Next, from the

perspective of attaining a well-designed study, what remain unknown is the relevant cellular elements which demarcates the stress-adaptive response of a cancer cell from normal ones. Given the intrinsic complexity and tremendously large number of intracellular elements, a pin-pointed study towards elucidating the distinctive stress survival of cancer cells should require a review of preceding literature on mechanotransduction with a view to isolate those elements which are maximally perturbed or influenced in response to fluid shear stress.

The shear stress is an inherited part of the microfluidic perfusion systems. For microfluidic perfusion culture in parallel plate flow chamber, the resulting parabolic flow profile yields the wall shear stress that is given by [39]:

$$\tau = 6\mu Q/h^2 w$$

Where, Q is flow rate, μ is fluid dynamic viscosity, w is flow channel width, and h is channel height. It is important to mention that the flow rate, channel height and width not only effect the shear stress developed in the microchannel, but also effect content of the soluble microenvironment including the nutrient delivery and secreted factors.

Generally, the laminar flow is taken into consideration while estimating the effect of shear stress on cellular behavior. In this regard, the Reynolds number (Re) can be used to determine whether the flow is laminar or turbulent. For parallel plate flow, is defined as [39]:

$$Re = \rho Q/\mu w$$

Where, ρ is the media density, Q is flow rate, μ is fluid dynamic viscosity and w is flow channel width. The flow is considered to be laminar if Re is small and considered as turbulent for greater Reynold's number.

1.5 The skin and its cellular organization

The skin is the largest organ in the body with an area of about 20 feet square. The skin has variety of functions including protection from physical, chemical and biological assaults including microbes, regulation of body temperature and permitting the sensations of heat, cold and touch. In mammals, the skin has three major layers: 1) the outermost layer of the skin which provides waterproof barrier, epidermis; 2) beneath epidermis there is dermis which is composed of connective tissues, hair follicles and sweat glands and act as a cushion against any kind of stress or strain; 3) the deeper subcutaneous tissue made of fat and connective tissue, hypodermis. The dermis and epidermis are separated from each other by a thin layer of fibres called the basement membrane which controls the cellular and molecular trafficking across the two layers. The dermis is mainly composed of fibroblasts which regulate the organization of the fibrillar dermal matrix; while the epidermis is composed of keratinocytes. The epidermis contains five layers including stratum basale, stratum spinosum, stratum granulosum, stratum licidum and stratum corneum (bottom to top). The cells in stratum basale are composed of basal keratinocytes which are considered to be stem cells of the epidermis. These basal keratinocytes proliferate and progressively move up the strata along with changes in shape and cellular composition. As the cells move up, the cells undergo differentiation and ultimately become anucleated. In this process, the keratinocytes become organized, forms adheren junctions, secrete keratin protein and lipids which forms essential components of ECM and contributes in its mechanical strength. Eventually, the keratinocytes shed from the stratum corneum within a span of 2 weeks.

1.6 Rationale of the work:

In recent years, much of the research had been focused on elucidating the influence of mechanical stresses on skin structure and its remodelling especially in relevance to wound healing applications [40]. The phenomenon of wound healing involves the proliferation,

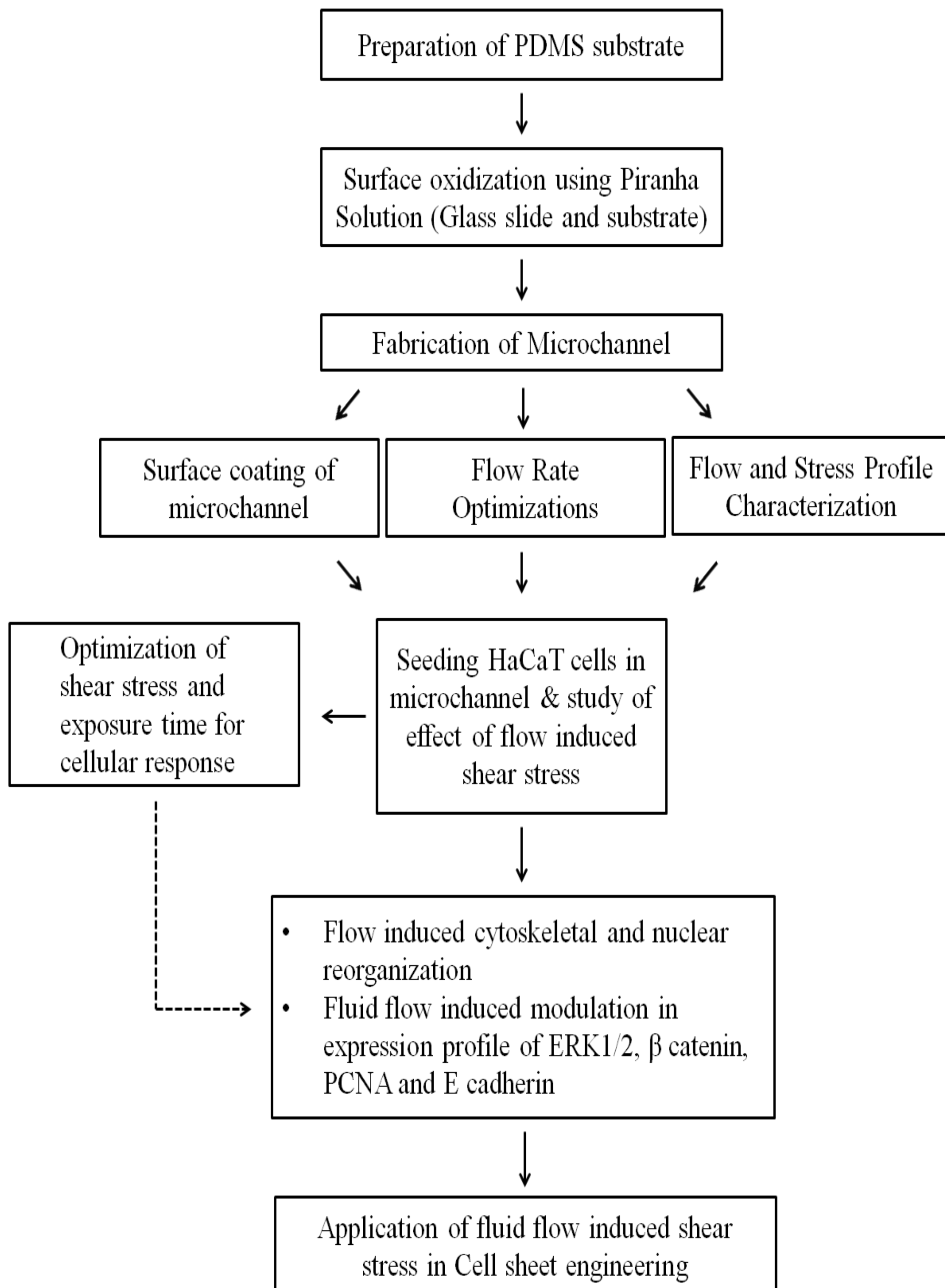
migration and differentiation of the epidermal keratinocytes, thus making them prime centre for analysis effects of these mechanical stresses. It has already been reported that the mechanical pressure induces the keratinocyte differentiation, while the application of mechanical stretching triggers the keratinocyte growth and proliferation. The mechanism proposed for such influence of mechanical stretching involves the activation of ERK1/2 via β1 integrin. Many researchers also propose the involvement of the calcium channels which gets activated under stress conditions [41-43]. It is important to mention that the cellular response under the influence of mechanical forces such as mechanical stretch, matrix stiffness follow integrin associated signal transduction pathway. However, none of the literature report the effect of fluid flow induced shear stress on epidermal keratinocytes. The fluid flow induced shear stress is considered as a crucial mechanoregulator and is known to influence the behavior of many cell types including osteoblasts, fibroblasts, endothelial cells, muscle cell [44-47].

Recently, the researchers have shown that fluid flow induced shear stress is capable of eliciting cellular response in the biliary and renal epithelial cells [48-49]. In this regard, it is quite interesting to explore the effect of shear stress on the epidermal keratinocyte behavior. However, these cells never influence the fluid flow, yet the flow induced shear stress could be utilized for cellular engineering applications.

1.7 Objectives of the work:

- Study of the role of fluid flow on cytoskeleton reorganization of HaCaT cells
- Understanding the mechanism of flow induced activation of cellular signaling pathways
- Potential application of flow induced cytoskeletal reorganization in epidermal cell sheet engineering

1.8 PLAN OF WORK



CHAPTER 2

MATERIALS & METHODS

2.1 Materials:

HaCaT cell lines were procured from NCCS, Pune. Dulbecco's Modified Eagle's Media (DMEM), Dulbecco's Phosphate Buffer Saline (DPBS), Trypsin-EDTA solution, Fetal Bovine Serum, Antibiotic-Antimycotic solution, MTT assay kit were purchased from Himedia, Mumbai, India. TRITC Phalloidin and DAPI were bought from Sigma-Aldrich, India. Syringe pump (model: SP-1000) was purchased from Ningbo Annol Medical Device Technology Co. Ltd.

2.2 Methodology:

2.2.1 Preparation of Microfluidic Channel and development of microfluidic setup:

A mixture of PDMS and crosslinker in 10:1 (w/w) ratio was used for microfluidic channel preparation. The mixture was poured, degassed, baked at 70 °C [50]. Thereafter, the microchannel substrate was peeled off the mould and wiped with 70% ethanol. The inlet and outlet ports were attached to the microchannel. The setup was assembled by connecting the microchannel with syringe pump using connecting tubings. The live imaging was carried out using phase contrast microscope (ZEISS PrimoVert). For continuous shear exposure, the complete setup was placed in CO_2 incubator at 95% humidity, 5% CO_2 and 37°C.

2.2.2 Cell Culture in Microfluidic channel:

HaCaT cell lines were cultured in DMEM supplemented with 10% FBS in a humidified (95%), CO_2 (5%) incubator at 37°C. The cells were harvested and seeded in microchannel at a concentration of $1x10^{6}$cells/ml. The seeded microchannel incubated in CO_2 (5%) incubator at 37°C and 95% humidity for next 24h.

2.2.3 Flow characterizations inside the microchannel:

The flow characterization inside the channels was carried out using PBS, incomplete and complete DMEM media using a syringe pump (SP-1000, Ningbo Annol Medical Device Technology Co. Ltd.). The characterizations were done at variable flow rates from 0.6ml/hr to 60ml/hr and the elapsed time was recorded. The real time fluid flow characterization in the microchannel was carried out using customized MATPIV code [51].

2.2.4 Optimization of flow rate and exposure time for cellular response:

The cells were exposed to the fluid flow using a syringe pump (model: SP-1000, Ningbo Annol Medical Device Technology Co. Ltd.). Initially, the variation in cellular morphology was observed using Phase Contrast Microscopy at 10X magnification (ZEISS PrimoVert). Static channel (without flow) was taken as control for examining the variations in the cells. A continuous flow (0.6ml/h, 6.0ml/h and 60ml/h) was applied in the microchannel placed in CO_2 (5%) incubator at 37°C and 95% humidity for 6 hours.

2.2.5 Flow induced cytoskeletal and nuclear reorganizations:

Further, the cytoskeletal - nuclear reorganization was analyzed using TRITC Phalloidin and DAPI staining after exposure to the shear stress. The static was taken as a control for the analysis. After the flow exposure, the cells were fixed using 4% paraformaldehyde and stained with TRITC Phalloidin and DAPI. Quantitative analysis of the same was carried out using MBF ImageJ software.

2.2.6 Immunocytochemistry:

For Immunocytochemistry, cells exposed to stress were fixed using 4% paraformaldehyde followed by permeabilization, blocking, treatment with primary and fluorophore-conjugated secondary antibodies and further visualization using confocal microscopy (Olympus IX 81 confocal microscope using Fluoview1000 system).

2.2.7 Cell Sheet Engineering:

The cell sheet inside the channel was formed by seeding cells at density of $4x10^6$cells/ml. Once the cells have adhered to the substrate sufficiently (8-10 hours), the media was changed at a regular interval of 3-4 hours until a proper cell sheet formed inside the microchannel. Thereafter, cells were exposed to shear stress and their morphology was characterized using field emission scanning electron microscope (FESEM) (Nova NanoSEM 450). Prior to visualization, the samples were sputter coated with gold using a sputter coater (Quorum Technologies, Q150R ES). The scanning was performed at 15kV.

2.2.8 Statistical Data Analysis:

Image processing was carried out using ImageJ and MATLAB (Mathwork) with custom derived algorithms. Statistical data were represented as Mean ± Standard Deviation. The P-value < 0.05 was considered as statistically significant after one way ANOVA analysis.

CHAPTER 3

RESULTS & DISCUSSIONS

3.1 Preparation of Microfluidic Channel:

The microfluidic channels were prepared using a mold developed by soft-lithography technique. In soft lithography, a negative mold is prepared by photolithography prepared using light sensitive photo resist. In brief, a fine coating of a light sensitive polymer is coated onto a solid glass or silicon substrate. When the coating is exposed high intensity ultraviolet light it hardens (in case of negative photoresist usually SU8 photoresist) [50]. Generally, the UV light is passed through a photomask some parts of which are darkened (desired microchannel shape). The unpolymerized coating of photoresist was rinsed using a particular developing solution, while the solidified fraction of photoresist is used as a negative mold. Finally, these molds were used to develop substrate for microchannels. For the preparation of microchannel substrate, Polydimethylsiloxane (PDMS) was used. Generally, dimethylsiloxane monomers and oligomers were mixed with polymerization catalyst. This mixture was poured into the mold and was placed at an elevated temperature to fasten the polymerization and solidification of PDMS. When poured into the mold, the PDMS acquires the complementary shape of the mold which provides a grove into the substrate after solidification. Subsequently, the substrate was peeled off the mold, oxidized by Piranha solution (to make the surface with groove; hydrophilic and suitable for bonding with the glass cover slip). Further the activated substrate was pressed against the glass coverslip and baked at 70°C for stable covalent bonding. Alternatively, a thin layer of PDMS-crosslinker mixture could be applied onto the PDMS substrate and then pasting it onto a glass slide followed by baking at 70°C. Once the microchannels were prepared, Inlet and outlet ports were made using a 20 gauge blunt end needle (Figure 2). In order to provide a continuous flow inside the microchannel, it was connected to a syringe pump using connecting tubings (Figure 3). The fluid flow inside the microchannel was optimized at various flow rates using fluid flow. The optimization of the flow was carried out by analyzing the volume passing through the channel per minute. Thereafter, the actual flow rate was compared with the theoretical flow rate (volume per unit time).

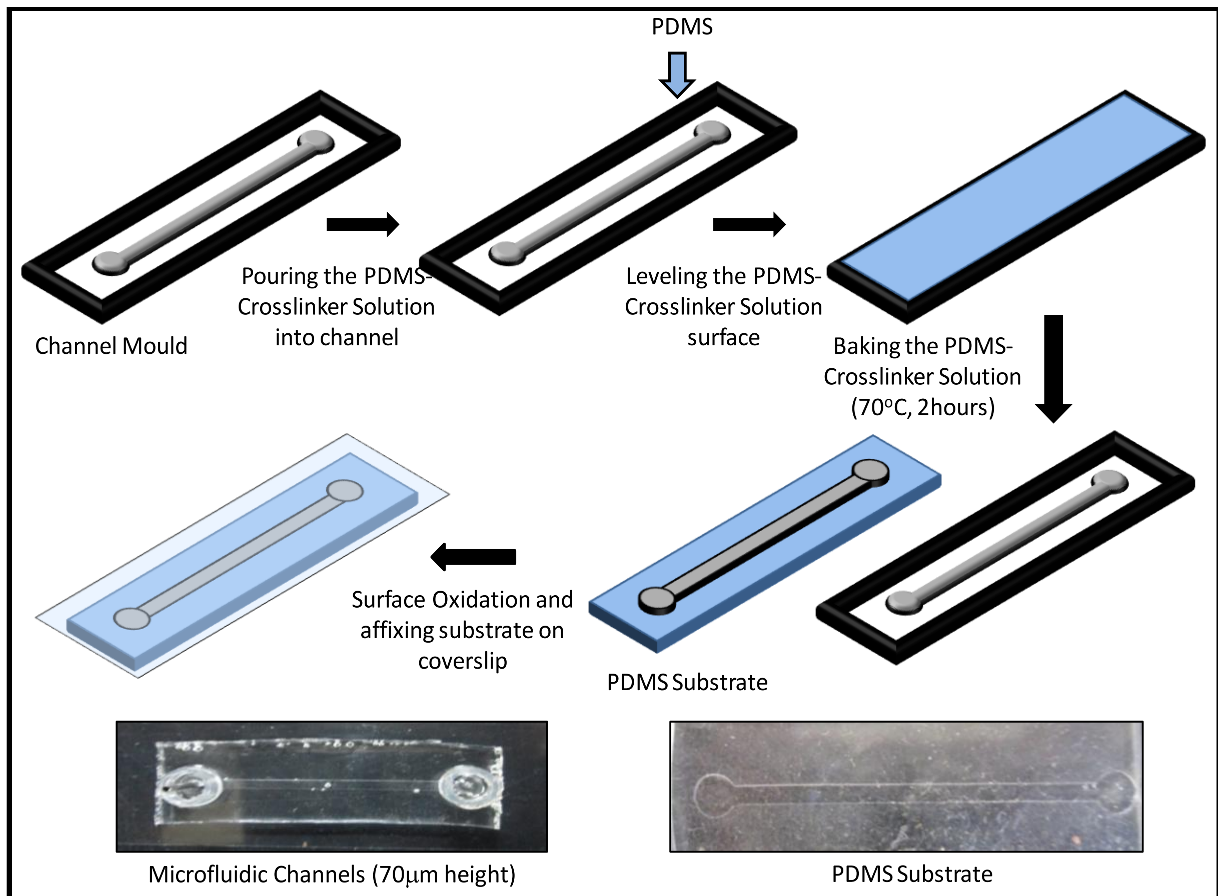


Fig. 2 Schematics for the preparation of the microfluidic channel

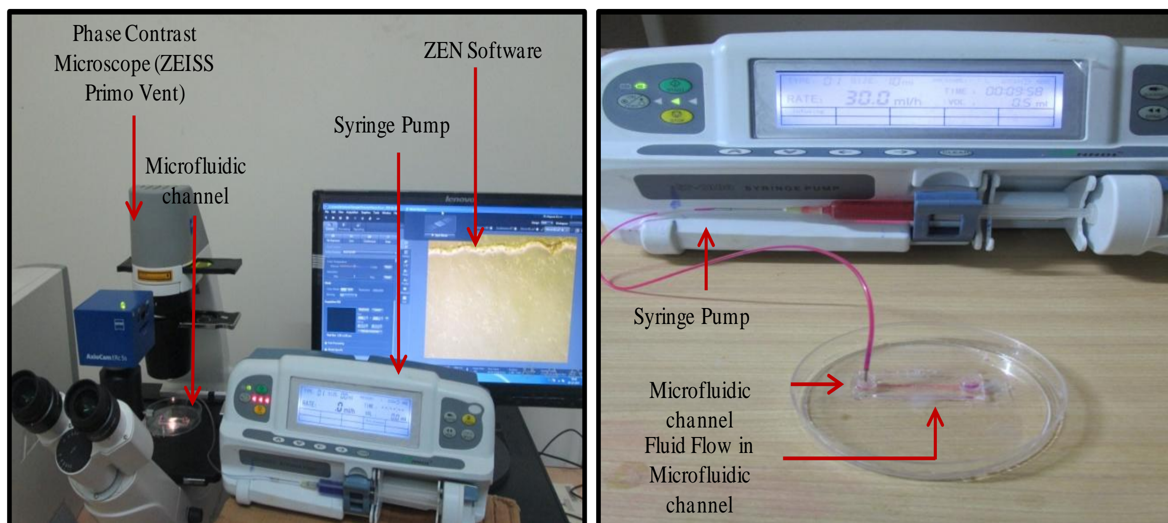


Fig. 3 Microfluidic setup for the real time visualization of the keratinocytes when exposed to shear stress

3.2 Cell Culture in Microfluidic channel:

In comparision to macro-system, cell culture inside a microchannel have been requires a considerable amount of attention. One prime reason for the same is the nutritional constrain in the microchannels. Owing to a very low volume inside the microchannel (< 7µl), the microconfinement, limits the amount of media available for each cell, thus exhausting the nutritional value of the media inside the microchannel very quickly. Moreover, absence of diffusive dilution results in the accumulation of the toxic metabolites which further leads to unfavorable cell culture conditions. To avoid such conditions, a fed batch process was preferred to replenish the media at regular time interval. The time interval for media replenishment was chosen depending upon the cell concentrations inside the channel. In our analysis, the microchannels were seeded with a cell concentration of 10^5cells/ml, which requires media replenishment at every 8-10 hour. Prior to cell seeding, the microchannels were coated with Fibronectin (50µg/ml) to promote greater degree of cell adhesion. For proper cell adhesion, the cells were incubated inside a humidified CO_2 incubator for around 24-30 hours. Thereafter, the cell seeded inside the microchannels were exposed to fluid flow induced shear stress. Prior to cell culture, the sterility of the microchannel also becomes a prime issue. The sterility of the microchannel was maintained by autoclaving at 121°C, 15psi for 20 minutes. Further, the channels were UV and ethanol sterilized. For proper cell survival, care was taken to remove residual alcohol from the microchannel by washing the channels at least thrice using phosphate buffered saline (PBS, pH 7.4) or serum free medium (Figure 4).

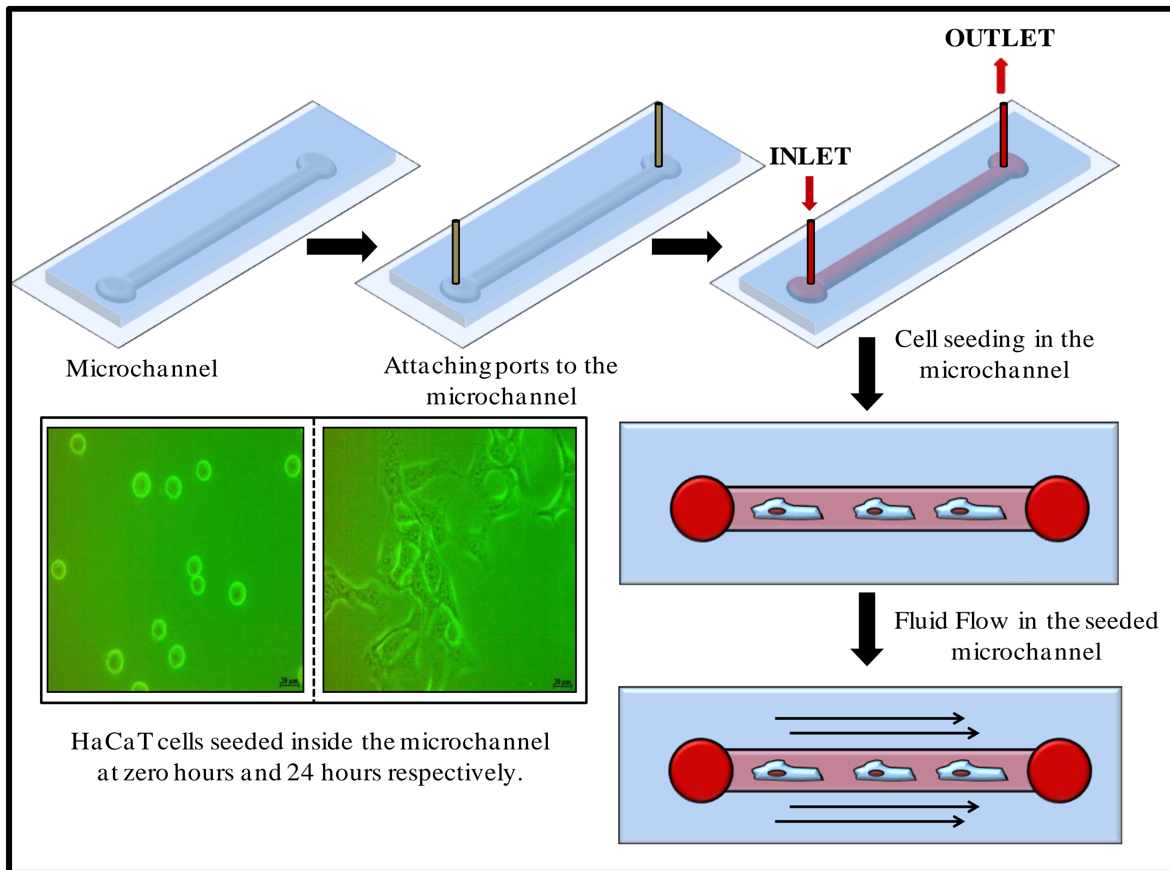


Fig. 4 Schematic representation of the cell seeding and exposure to fluid flow induced shear stress in microfluidic channel

3.3 Flow characterizations inside the microchannel:

The real time flow characterizations inside the microchannel demonstrated that axial average velocity profile obtained from this analysis showed certain deviations from the ideal parabolic velocity profile which happens due to (1) failure of system to resolute between two subsequent beads at that experimental frame rate, (2) roughness of the channel boundary. However, the coherence nature of the velocity field implied possible application of this technique with further modifications (Figure 5).

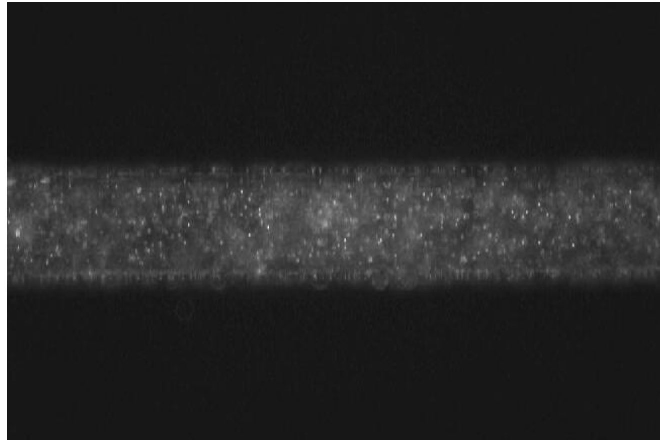


Fig. 5 The microscopic view field (4X) of MATPIV experiment.

3.4 Optimization of flow rate and exposure time for cellular response:

Physiologically, most of the cells are exposed to the interstitial flow through the matrix confinements. This interstitial fluid flow posses a front velocity ranging within 0.1-10 μm/s and a corresponding wall shear stress within 0.1-10 dyne/cm^2. It is generally observed that upper limit of the shear stress is capable of influencing the cellular physiology, morphology and proliferation; while at lower flow velocities the shear stress is negligible and was incapable of eliciting a cellular response. However, the influence of the fluid flow induced shear stress has not reported for the skin keratinocytes. Thus, it becomes quite essential to optimize the quantitative parameters including the amplitude of shear stress and the time of exposure that can elicit a keratinocyte cellular response. Our results suggested that the shear stress of 0.06, 0.6 and 6 dyne/cm^2 elicited a variable cellular response. A qualitative analysis of the phase contrast demonstrated that higher shear stress induced cellular elongation. However, the shear stress of 0.06 dyne/cm^2 induced a greater degree of cellular spreading. It is important to mention that keratinocyte exposure to a higher shear stress of 6 dyne/cm^{-2}, resulted in cellular damage (Figure 6) [52]. The keratinocyte response at 0.06 dyne/cm^2 were quite interesting and were further used to optimize the time of exposure of the shear stress to elicit such response. From the analysis it was observed that an exposure of about 6 hours was

sufficient enough to cause keratinocyte spreading. Thus, the amplitude of shear stress and the time of exposure were kept constant to 0.06 dyne/cm^2 and 6 hours respectively for the complete study (Figure 7).

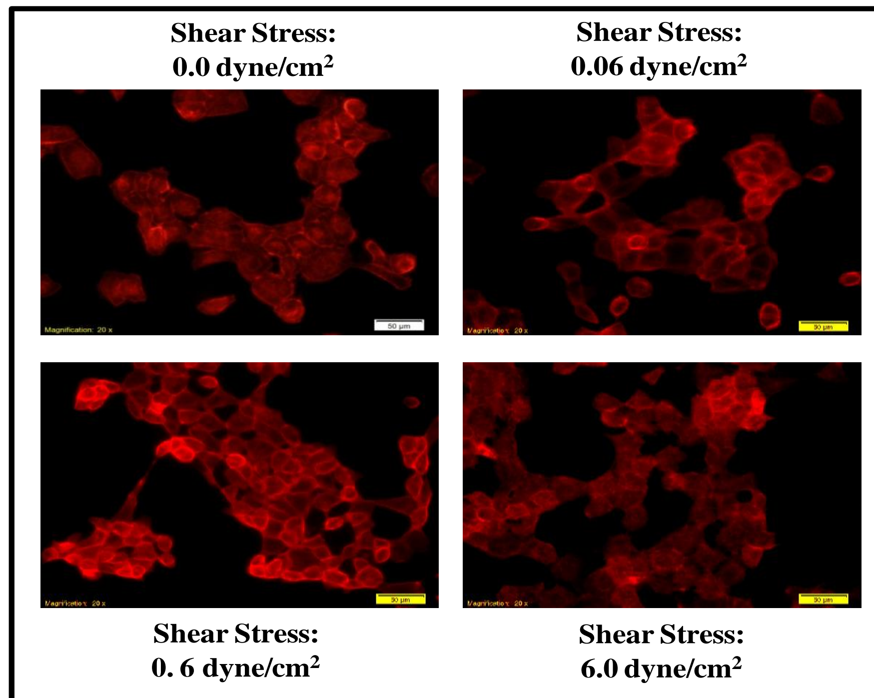


Fig. 6 Representative images of cytoskeletal reorganization with variation in the amplitude of shear stress (0.06 – 6.0 dyne/cm^2) Static was taken as control for the analysis.

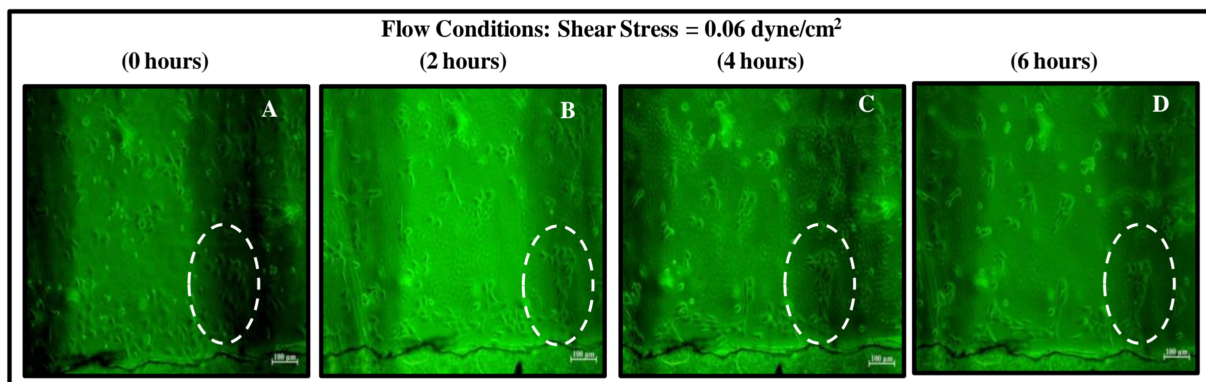


Fig. 7 Time Dependent Variations in the cellular morphology at shear stress of 0.06 dyne/cm^2 (flow rate: 0.6 ml/hour).

3.5 Flow induced cytoskeletal and nuclear reorganizations:

A number of researcher have reported that the fluid flow induced shear stress results in cytoskeletal reorganization in variety of cells including bone cells, endothelial cells, fibroblasts, skeletal muscle cells etc [53-56]. The major cytoskeletal proteins of the cells include that actin filaments and intermediate filaments. It is generally considered that the cellular cytoskeleton undergo reorganization in order to resist the stress condition (Figure 8). Our results suggested that under influence of stress, the actin microfilaments became denser. However, a higher shear stress of 6 dyne/cm^2 resulted in the disruption of actin fibres and thus the cellular damage (Figure 9). It is important to mention that under static conditions, the actin cytoskeleton was distributed randomly in the form of mesh. However, under stressed condition the actin was more concentrated on the cellular boundaries with the formation of dense stress fibres. A critical analysis of cellular and nuclear area suggested that with an increase in the shear stress the cellular and nuclear area increased significantly. On the other hand, the increase in the shear stress also resulted in greater degree of cellular elongation. Interestingly, exposure of cells to the shear stress of 0.06 dyne/cm^2 resulted in the increase of cellular and nuclear area with a decrease in the cellular and nuclear elongation. It is important to mention that under a shear stress of 0.06dyne/cm^2, there exists 1.34 folds increase in the cellular spreading. This clearly suggests that a lower shear stress of 0.06dyne/cm^2 exhibit a significant influence on the keratinocytes with greater spreading and lower elongation.

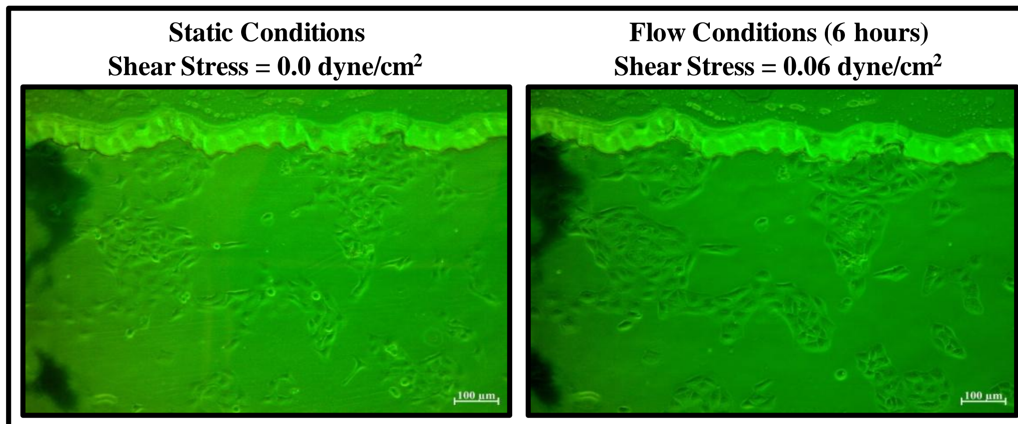


Fig. 8 Changes in the keratinocyte morphology after exposure to shear stress of 0.06 dyne/cm^2. Static was taken as control for the analysis (Phase contrast microscopy)

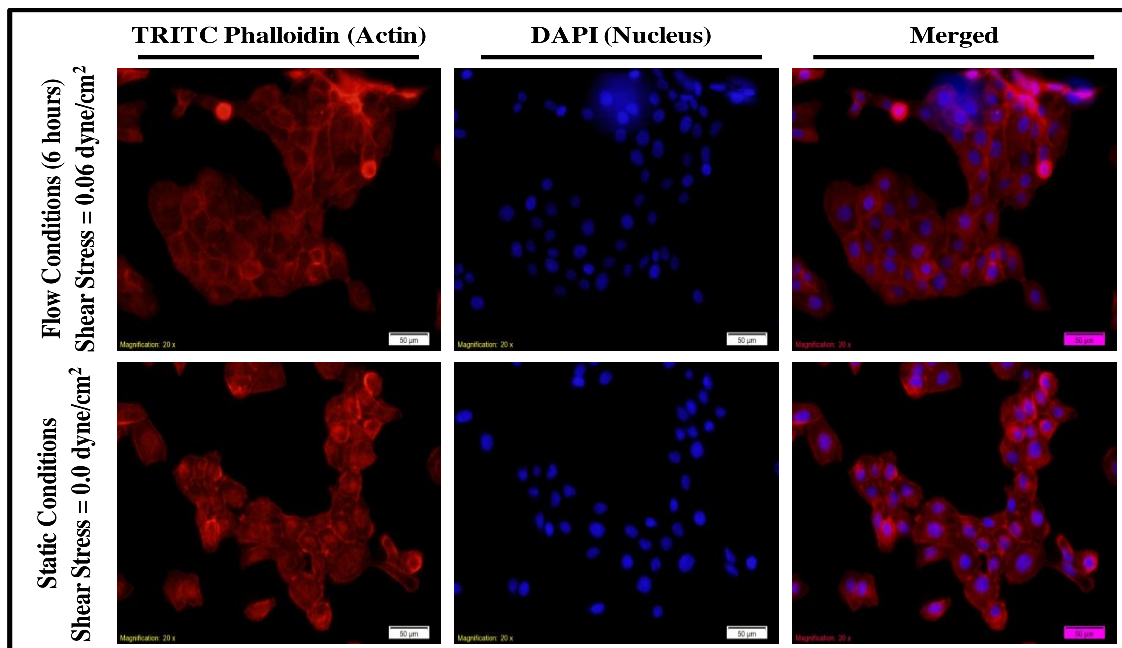


Fig. 9 Variations in cytoskeletal and nuclear reorganization on exposure to shear stress of 0.06 dyne/cm^2 confirmed by TRITC Phalloidin (actin) and DAPI staining (nucleus). Static was taken as control for the analysis.

3.6 Fluid flow induced modulation in expression profile of ERK1/2, β catenin, PCNA and E cadherin:

The integrins are involved in the cell-substrate adhesion and is considered as an important mechanotransducer [6]. It recruits various adhesion-associated linker proteins on their cytoplasmic domain to form focal adhesion complexes. These focal adhesion complexes have both structural and biochemical functions and are known to play a critical role in mechanotransduction events [57]. The adhesion associated proteins include talin, vinculin, p130Cas and focal adhesion kinase (FAK). The changes in the integrin conformation activate these adhesion associated proteins leading to changes in cytoskeletal protein arrangement [58]. Amongst all, Focal adhesion kinases (FAK) are considered to be a crucial for integrin stimulated signaling pathways which leads to the activation of Ras/Raf/MEK/Erk1/2 and PI3K/Akt signaling cascades. Many researchers have reported that FAK influences the cellular adhesion and spreading [59]. Our results demonstrated that under the influence of shear stress a greater activation of Erk1/2 (Figure 10). The increased activation of ERK1/2 may be a consequence of greater activation of FAK owing to greater cellular spreading. Also, ERK1/2 (pT202/Y204) may increase the phosphorylation of GSK3β resulting in increased accumulation of β-catenin in the cytoplasm [60-61]. These events together regulate the nuclear translocation of β catenin, thereafter binding with LEF/TCF and thus transcription of target genes involved mainly involved in cellular proliferation and differentiation [60-62]. Our results demonstrated that under influence of fluid induced shear stress higher cellular accumulation and nuclear localization of β-catenin was observed (Figure 11). The greater localization of the β-catenin to nucleus will increase the proliferative activity of the keratinocytes. In order to validate that we analysed the expression and localization of PCNA. As expected, higher PCNA expression along with greater nuclear localization was observed when the cells were exposed to shear stress of 0.06 dyne/cm^2 (Figure 12).

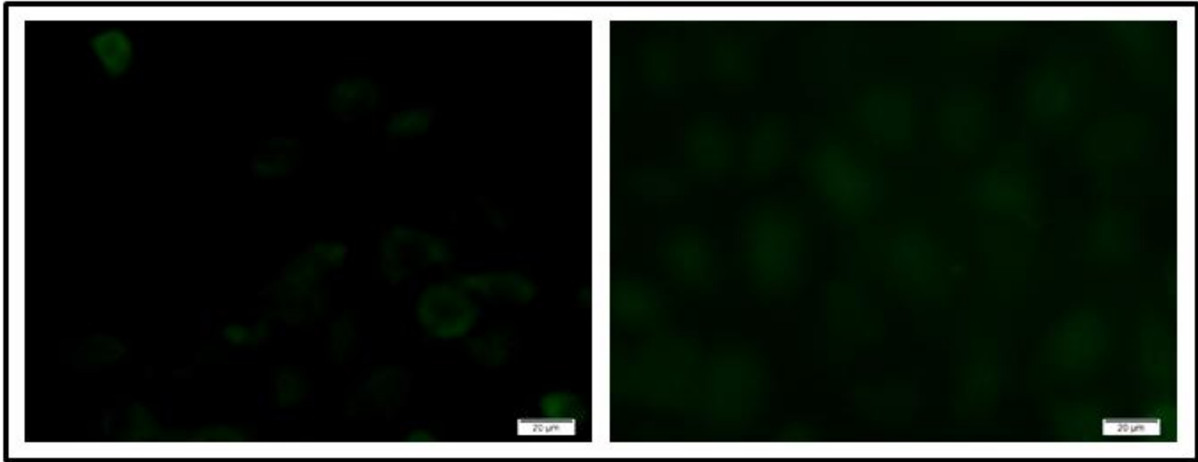


Fig. 10 Representative images of ERK1/2 (pT202/Y204) expression profile of HaCaT cells under shear stress of 0.06 dyne/cm^2. The static was taken as a control for the analysis (confocal microscopy).

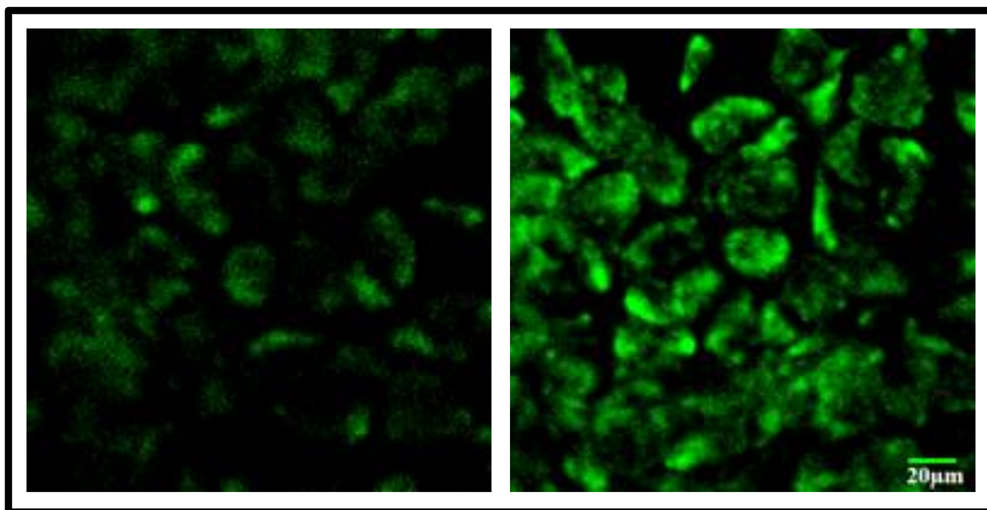


Fig. 11 Representative images of β-Catenin expression profile of HaCaT cells under shear stress of 0.06 dyne/cm^2. The static was taken as a control for the analysis (confocal microscopy).

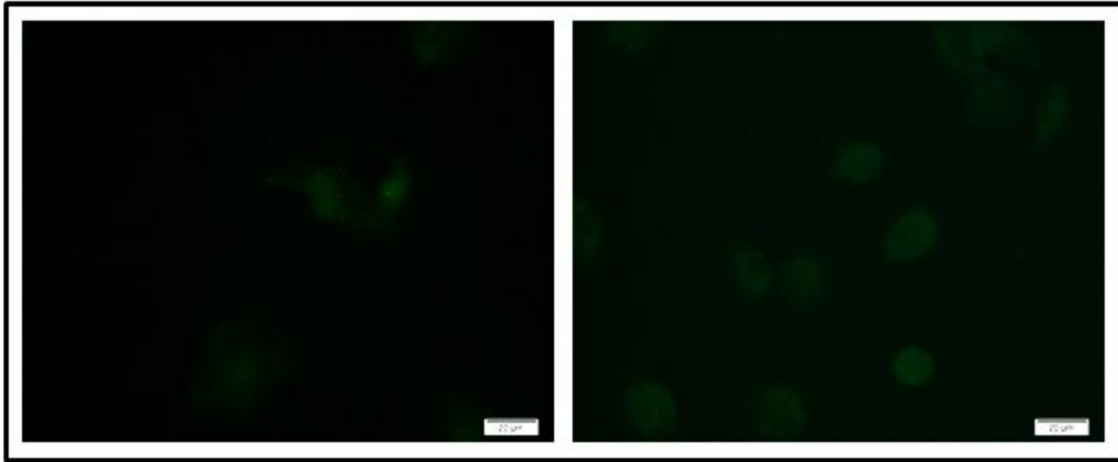


Fig. 12 Representative images of PCNA expression profile of HaCaT cells under shear stress of 0.06 dyne/cm^2. The static was taken as a control for the analysis (confocal microscopy).

On the other hand, the cell-cell contact also plays a crucial role in modulating overall cellular behavior. Cadherins are also a large family of calcium-dependent cell adhesion proteins known to be involved in cell–cell adhesion and suitable candidates for the mechanosensation and mechanotransduction [63-64]. The intracellular domain of cadherins allows them to anchor to the cytoskeleton by vinculin, α, β and γ catenin, which form a multimeric protein complex [65]. Our results demonstrated that under the shear stress the HaCat cells respond by formation of additional adheren junctions. A critical analysis of the confocal images demonstrated higher greater number of actin bridges under stressed condition in comparision to static control. These actin bridges are considered as important for the localization and stabilization of the E-cadherins and subsequently facilitate the formation of adheren junctions [66]. However, it is important to mention that immunofluorescence images demonstrated intracellular presence of E-cadherin (Figure 13). A plausible explanation of the same may be the cadherin reorganizations in response to the cell spreading. Such reorganization under influence of shear stress may be a result of E-cadherin internalization as reported by Lawler *et al* [24]. Moreover, Cytochalasin D treatment for 30 minutes significantly reduced the

earlier observed cellular spreading on exposure of shear stress of 0.06 dyne/cm^2. A critical analysis showed that there exists an insignificant difference in the cellular spreading between the static and stress exposed keratinocytes after Cytochalasin D treatment (Figure 14). This clearly suggests that the actin cytoskeleton play a crucial role in modulating the overall keratinocyte behavior under influence of shear stress.

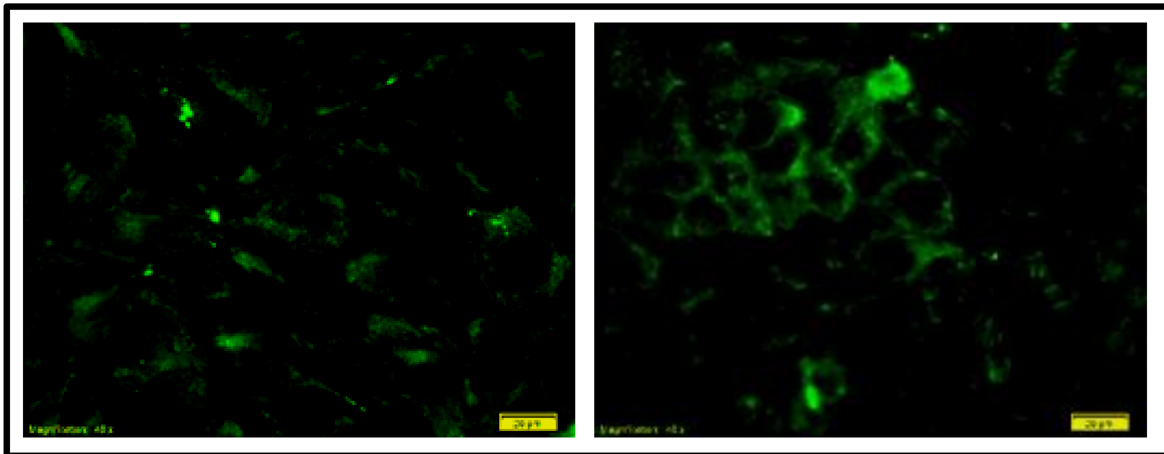


Fig. 13 Representative images of E-Cadherin expression profile of HaCaT cells under shear stress of 0.06 dyne/cm^2. The static was taken as a control for the analysis.

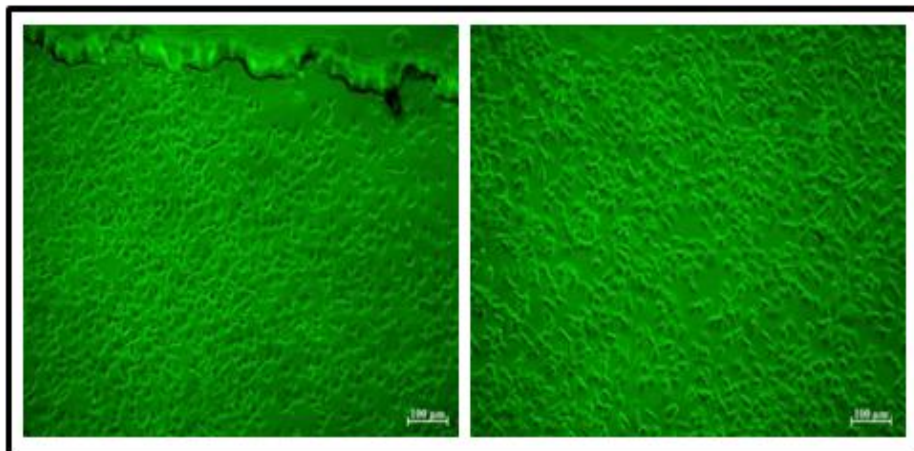


Fig. 14 The cellular morphology after Cytochalasin D treatment under shear stress of 0.06 dyne/cm^2. The static was taken as a control for the analysis (Phase Contrast microscopy).

3.7 Application in Cell Sheet Engineering:

In order to decipher the application point of view of fluid flow induced shear stress on HaCaT skin keratinocytes, a confluent cell layer was formed inside the microchannel and estimated for cellular characteristics using FEI SEM. The results demonstrated that after exposure to the flow the cells flattened more in comparision to the static system and the cells were closely packed, thus indicating a potential application of shear stress in cell sheet formation (Figure 15).

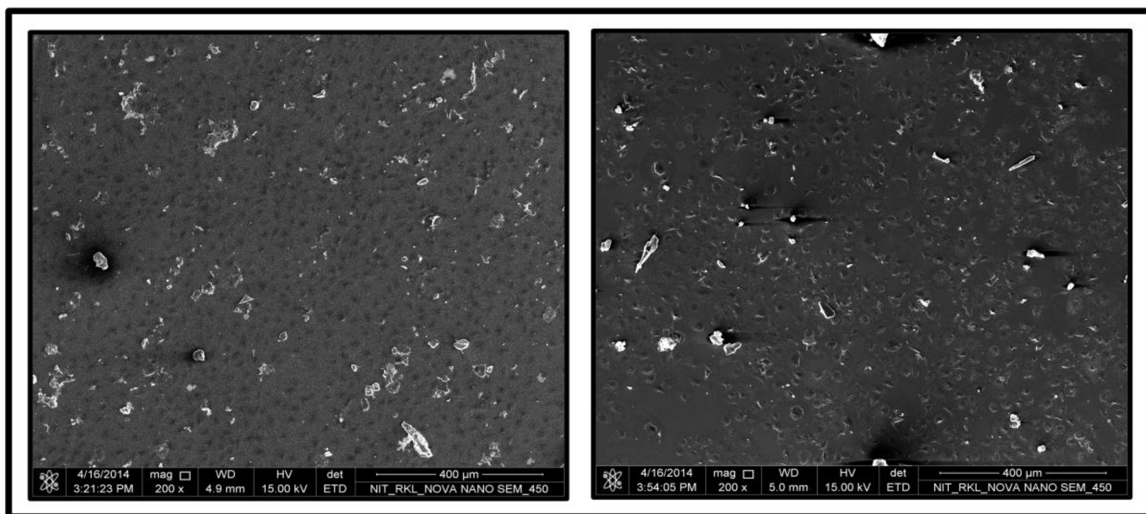


Fig. 15 Scanning Electron micrograph of the confluent cell layer after exposure to shear stress of 0.06 dyne/cm^2. Static was taken as control for the analysis.

CHAPTER 4

CONCLUSION

4.1 Conclusion

In the present investigation we have successfully demonstrated that the fluid flow induced shear stress is a mechanoregulator of the epidermal keratinocytes. We showed that shear stress could effectively modulate the keratinocyte behavior accounted in terms of its morphology, cytoskeletal and nuclear reorganizations and its protein expression. We hereby propose that the cellular response to shear stress follow ERK1/2 mediated signal transduction pathway via β1 integrin. It is important to mention that the cellular response under the influence of mechanical forces such as mechanical stretch, matrix stiffness also follow the same integrin associated signal transduction pathway. However, these cells never influence the fluid flow, yet the flow induced shear stress could be utilized for cellular engineering applications.

4.2 Future Prospective

- Further investigation and validation of the immunocytochemistry results by western blotting.
- Optimization of the conditions to form a cell sheet inside the microchannel.
- Mechanical characterization of the cell sheet exposed to the fluid flow induced shear stress.

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