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Author: Amish Asthana William S. Kisaalita

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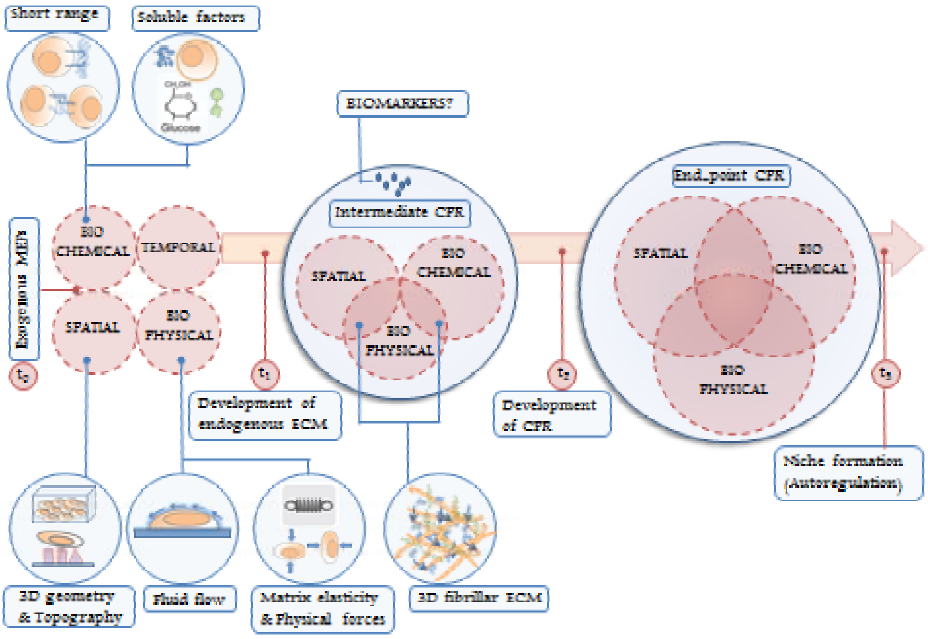
Graphical Abstract

A schematic depiction of a total microenvironment (3D culture) supporting the formation of microtissue that exhibits 'complex' physiological relevance (CPR) or better emulation of the *in vivo* tissue functionality in a manner not possible in 2D cultures.



Highlights:

- A case is made for the need for complex physiological relevance (three-dimensionality) biomarkers expressed early in culture
- Transcriptomic and proteomic upregulation of cytokine observations in 3D cultures are summarized
- A sound molecular basis (*Ras/Raf/ERK* signaling pathway) for the upregulation of cytokines in 3D points to cytokines as the suitable three-dimensionality biomarkers
- Identification and validation of the cytokine three-dimensionality biomarkers is advocated

# Molecular basis for cytokine biomarkers of complex 3D microtissue physiology *in vitro*

Amish Asthana and William S. Kisaalita*

Cellular Bioengineering Laboratory, College of Engineering, Driftmier Engineering Center, University of Georgia, Athens GA 30602, USA

*Corresponding author: Kisaalita, W.S. (williamk@engr.uga.edu).

*Keywords*: Biomarkers; 3D; three-dimensionality; cytokines; tissue engineering; drug discovery; material discovery; cell-based biosensors.

*Teaser*: Cytokines make excellent early biomarkers for complex 3D microtissue physiology *in vitro*. They should be a powerful tool in establishing cell–cell and cell–material interactions in microenvironments that support physiologically more-relevant microtissue formation when validated.

‘Physiologically more-relevant’ claims are readily made for cells cultured on any surface or in a scaffold that provides loosely defined 3D geometry. A set of tools to measure culture ‘3D-ness’ more accurately are needed. Such tools should find applications in fields ranging from high-throughput identification of substrates for tissue engineering and regenerative medicine to cell-based screening of drug candidates. Until now, these fields have not provided a consensus for the most promising place to initiate the search. Here, we review recent advances in transcriptomic, proteomic, inflammation and oncology-related pathways, as well as functional studies that strongly point to cytokines as the most likely compounds to form the missing consensus.

**Introduction**

The definition of 3D cell culture has recently been extended from simple spatial organization to providing a complete microenvironment that leads to the formation of a complex physiologically relevant microtissue. This new definition can be translated to mean better emulation of *in-vivo*-like functional competence in a way not achievable in monolayer cultures [1]. Interestingly, the crucial components of this microenvironment can be expressed in a 3D Cartesian coordinate system (Figure 1), with the following microenvironmental factors (MEFs) or three-dimensions of: (i) biochemical or chemical configuration; (ii) temporal dimensions and spatial (geometric 3D) architecture; and (iii) force and substrate physical properties. The three MEFs are well supported by recent literature [1–3]. In the following four paragraphs, before proceeding, we provide four compelling reasons why searching for three-dimensionality biomarkers in required.

First, if the resultant phenotypic characteristics are different between cells growing in 2D and those grown on any platform that provides a loosely defined 3D architecture, either at the nano- or micro-scale or their combinations, they are deemed to be physiologically more relevant. Apart from the 3D matrix adhesion, initially proposed by Cukierman[s1] *et al.* [4] as a possible 'diagnosis' or indication for the three-dimensionality of a culture, the area of tissue engineering has not provided the knowhow basis on which these claims could be validated. As such, a consensus for the 3D state of a culture and the complex physiological relevance associated with it should be established.

Second, hit materials can be rapidly screened for future development via high-throughput combinatorial approaches. A library could be generated with information on polymers or other scaffolding substrates [5,6] useful in tissue engineering or cell-based drug discovery

applications, providing 3D assays are available. The advancement of these assays or biosensors can potentially be governed by a cell–substrate interaction outcome [7]. As previously pointed out, three-dimensionality [s2]biomarkers would provide the intellectual basis for material discovery platform development, where interactions with a substrate that produces cells that mimic *in-vivo*-like competence are desired [7]. Figure 2 shows an illustration of a theoretical framework in which scaffold substrate discovery can be carried out in a HTS [7] format. As shown in Figure 2a,b, poly(desamino tyrosyl-tyrosine ethyl ester carbonate) (pDTEc) and poly (desamino tyrosyl-tryrosine octyl ester carbonate) (pDTOc) have a structurally similar backbone, but different side-chains (i.e. ethyl on pDTEc and octyl of pDTOc). Because of this, they show different properties, which lead to variations in cells grown on films (2D) of the polymers and their composites. When cultured on pDTEc, cells displayed enhanced adhesion, spreading and proliferation in comparison with pDTOc [8,9]. If a balance between differentiation and proliferation is needed, then an optimum polymeric composition that satisfies the requirement might exist and readily be found using HTS with a polymer blend combinatorial scaffold library. The library can be constructed using a fluid handling instrument represented by a dual syringe pump system (Figure 2c) that would generate arrays of porogen-leached scaffolds of varying polymeric blends (Figure 2d). In the proof-of-concept study of the system shown in Figure 2, Yang *et al.* [6,7] used Fourier transform infrared spectroscopy (FTIR) to verify scaffold polymer mixtures. Extending such a study to the question of how well the polymer blends support the three-dimensionality of cultures within requires biomarkers measurable in HTS readouts.

Third, it is essential to reduce the costs of 3D platforms to increase their accessibility for HTS applications; simplification of the platform without affecting the physiologically relevant behavior of the cells can only be achieved with validated biomarkers. In recent work, the

definition of three-dimensionality in cell culture has been extended to provide a complete microenvironment that leads to the formation of a complex physiologically relevant microtissue or better emulation of *in-vivo*-like functional competence in a way not achievable in monolayer cultures [1] (see Figure 1 for more detailed illustration). The platform simplification can be achieved easily if the physiologically relevant outcome can be measured in terms of three-dimensionality biomarkers, as elaborated by Lai *et al.* [10]. As the architecture of the platform is simplified, it will be feasible, using validated biomarkers, to know when the trajectory toward complex physiological relevance (CPR) outcomes is being affected.

Fourth, CPR is a phenomenon that is generally expressed late in culture and is often associated with a combination of structural and functional attributes that are not quantitative. A biomarker that might be quantitative and expressed early in culture can act as an early indicator of the trajectory toward CPR outcomes. Additionally, different techniques are typically employed to detect CPR in different tissue types and they are often incompatible with HTS. Therefore, a ubiquitous biomarker expressed early in culture would provide a single assay that can[s3] predict CPR outcomes in cells derived from many tissue types. Taken together, the field of 3D culture requires validated biomarkers. The elephant in the room issue is whether such a thing exists. In this review, we bring together evidence from transcriptomic, proteomic, inflammation and oncology-related pathways, as well as cellular functional studies that strongly suggest that cytokines are the most likely entity to provide the badly needed biomarkers.

**Timing of CPR and screening studies**

Differences in resulting cell phenotypes between 2D and 3D platforms are necessary but not sufficient for suggesting CPR. It is therefore necessary to show conclusively that cells cultured

on 3D platforms are emulating the functional and/or structural outcomes observed *in vivo*. The well-established or provisional CPR outcomes for cells belonging to the three types of tissues of major interest in preclinical drug discovery (i.e. epithelial, cardiac and neuronal) have been discussed in detail by Asthana and Kisaalita [11]. Although structural and/or functional CPR outcomes can be used to establish and validate three-dimensionality, there are additional questions or drawbacks beyond the late-in-culture expression that has been mentioned already. For example, a CPR outcome can be considered an endpoint measurement, suggesting that the culture has been on a trajectory, prior to this point, toward this desired *in vivo* emulation state. A natural question to ask is: when is the most appropriate time to use the microtissue in screening studies – before, after or at this point? It is well known that the viability of microtissues *in vitro* has a limited time. This raises the question of how long after observing CPR outcomes is the microtissue suitable for screening studies? Assuming a cytokine expression profile early in culture accurately predicts CPR outcomes expressed later in culture, are the resulting microtissues suitable for screening studies before CPR outcome expression? Answers to the above questions are crucial to the advancement and utilization of complex physiologically relevant 3D cell-based assays. To answer these questions or to establish the optimal performance time for a microtissue, with respect to meaningful assay results, biomarkers of three-dimensionality that reliably predict CPR outcomes and are expressed early in culture are a must-have.

**Cohesivity: microtissue formation and cytokine production**

Living organisms have the ability to perceive their environment and respond to changes in it. Cells, being the building blocks of an organism, are also endowed with this power. Their

surrounding, being comparable to their own size, is termed the microenvironment and cells are affected by changes in it. If the cells are transitioned from a monolayer culture to a 3D environment how do they perceive this change? Cells in 3D find themselves in the vicinity of homotypic neighbors leading to the formation of a loosely bound aggregate. A similar scenario is encountered *in vivo* during avascular tumor progression, early stages of inflammatory wound healing and development. These phenomena are similar in nature and are controlled by the same molecules: cytokines [12]. *In vitro*, depending upon their nature (malignant, primary or stem), the cells cultured in 3D relate to any of these phenomena and therefore an increase in their cytokine expression is physiologically justified. Cytokines are soluble low molecular weight extracellular protein mediators that typically act at short range between neighboring cells. They have important roles in intercellular regulation and mobilization of cells engaged in innate and adaptive inflammatory host defenses, cell growth and death, differentiation, angiogenesis, development and repair processes [13]. They have been studied extensively for their role in inflammation, tumor progression and normal development [12–14]. Cytokines and their associated receptors provide major signals for essential processes. Abnormalities associated with them, their receptors or the signaling pathways they affect are involved in a wide array of diseases, particularly by promoting and perpetuating inflammation. Based on the structural homologies of their receptors, cytokines have been assigned to various family groups and can be broadly classified into: colony stimulating factors, interleukins (IL), interferons, transforming growth factors (TGFs), tumor necrosis factors (TNFs), platelet-derived growth factors (PDGFs) and chemokines. They act on cells expressing complementary receptors in autocrine and paracrine manners [15]. They can control their own production as well as that of the others by initiating a feedback loop [16–18]. Cytokine production and function is generally governed by

the transcription factors nuclear factor (NF)-κB [19] and activator protein (AP)-1 [20] because they have binding sites in the promoter region of most of the cytokine genes. The extracellular-signal-regulated kinase (ERK) [21] and the Janus kinase/signal transducers and activators of transcription (JAK/STAT) [22,23] pathways have also been widely implicated in their regulation.

Along with the downregulation of cytokines, discussed in a later section, another intriguing absence in the case of 2D monolayers is that of cell adhesion molecules (CAMs). This is substantiated by the microarray analysis done on many established cell lines and tissue-derived cells in which CAMs were found to be downregulated in conventional cultures [24]. CAMs are transmembrane receptors found on the surface of the cell and are composed of three domains: (i) an extracellular domain that enables interactions either with similar CAMs (homophilic binding) or with different CAMs or the extracellular matrix (heterophilic binding); (ii) an intracellular domain that facilitates interactions with the cytoskeleton; and (iii) a transmembrane domain. CAMs can be classified into five protein families: immunoglobulin (Ig) superfamily (IgSF CAMs), integrins, cadherins, selectins and the lymphocyte homing receptor. Their structure and function have been reviewed in detail elsewhere [25]. In 3D cultures, formation of aggregates or microtissues is brought about by cell–cell and cell–ECM (extracellular matrix) interaction through CAMs [26]. Traditionally, integrins have been associated with cell–substrate adhesion whereas cadherins form cell–cell adherens junctions [25]. However, recent studies carried out on CHO cells [27] and fibroblasts [28] have shown that integrins play a part in forming cohesive cell–cell bonds in a 3D microenvironment leading to the formation of a microtissue. Cohesivity seems to be brought about by integrin binding to the fibronectin (FN) matrix assembly [29] present in the ECM of 3D cultures, but absent in monolayers. Cellular crosstalk mediated by

integrins and the subsequent intracellular pathways that they invoke might be related to the upregulation of cytokines in 3D. This is the main premise behind cytokines as the most likely family of compounds to provide the missing three-dimensionality biomarker consensus. In the remainder of this review, we provide more-detailed experimental evidence to support this proposition. We have organized our presentation thus: (i) 2D–3D comparative transcriptomic and proteomic studies; (ii) integrin–FN studies: and (iii) signaling pathways involved as an overarching view that integrates the other experimental evidence.

**2D–3D culture comparative transcriptomic–proteomic upregulation of cytokines**

Recently, many studies have shown that when cells are grown in a 3D culture their cytokine levels are elevated as compared with the traditional monolayer cultures (Table 1). We compared neural progenitor (NP) cells grown on 2D substrates, 3D porous polystyrene scaffolds and as 3D neurospheres (*in vivo* surrogate) with respect to transcriptomic expression using the Human Whole Genome U133 plus 2.0 GeneChip Expression Analysis (Affymetrix, Santa Clara, CA) [14]. The expression data are available on the GEO site as Series GSE13715[s4]. An upregulation in the expression of cytokines as a group in 3D and neurospheres was observed. The numbers of probe-sets that were elevated in 3D and neurospheroid culture conditions were 40 and 91, respectively. The difference in the number of upregulated probe-sets might be the result of an inability to regulate the diameter of neurospheres. Many neurospheres were observed to be bigger than the pore size of the 3D scaffolds (e.g. three-times the maximum pore diameter of 100 μm) [30]. The core of the microtissue can experience hypoxia in large neurospheres, to the extent that genes not observed in 3D are upregulated. For example, macrophage inflammatory protein (*MIP*)-*2* gene, induced by hypoxia [31], was found to be elevated in neurospheroids but not in

3D conditions in our study. A group of 13 cytokines including angiopoietin-like 7 (ANGTL7)/CDT6, ARMET[s5]/mesencephalic astrocyte-derived neurotrophic factor (MANF), bone morphogenetic protein 8B/osteogenic protein 2 (BMP8B/OP2), CCL13/monocyte chemotactic protein-4 (MCP-4), fibroblast growth factor 5 (FGF5), ghrelin (GHRL)/obestatin, IL-11, IL-1B/IL-1F2, nephroblastoma overexpressed (NOV)/IBP-9, platelet-derived growth factor subunit B (PDGFB), stanniocalcin-1 (STC1), transforming growth factor alpha (TGFA) and vascular endothelial growth factor A (VEGF-A) were commonly elevated in cells grown in polystyrene scaffolds and neurospheres. As such, any or a combination from this list has the potential to serve as biomarkers of three-dimensionality.

We particularly focused on the above genes because there was less likelihood of them being upregulated as a result of hypoxic conditions that could be present in neurospheroids [s6]but not in 3D conditions. The functional classification of these cytokines suggested an underlying theme of development, which is physiologically relevant because progenitor cells in a 3D culture *in vitro* try to emulate embryonic development. The result of this study is in agreement with many other transcriptomic and proteomic studies where cytokines have been shown to be elevated in 3D cultures. These studies have been conducted on many different cell lines spanning various cell types including primary cells, fibroblasts, multi and pluripotent stem cells and cancer cells grown on different 3D platforms. The results are summarized in Table 1. As shown, despite the fact that there are no neural cells in the studies cited in Table 1, identical (IL-11) or related (ANGTL7, IL-1B and VEGF-A) cytokine upregulation from our study has been reported by others from different cell types. In a recent review discussing how cells know where they are, Lander [32] has emphasized the importance of morphogen diffusion gradients. An upregulation of cytokines would result in a change in the diffusion gradient. Because many cytokines (e.g.

BMP8B, PDGFB) are well known morphogens, this change in the diffusion gradient results in different cellular activity in 3D compared with 2D cultures. Taken together, this shows that cytokines have the potential to validate the 3D platforms with respect to emulating the *in vivo* microenvironments. Cytokines are suitable as biomarkers because they are secreted in the culture media and can be quantified without lysing by ELISA [33]. This will not be the first instance for the proposed usage of cytokines as biomarkers. Owing to their robustness and versatility, cytokines have been proposed extensively to be used as markers for early detection of head, neck [34] and ovarian cancers [35], Alzheimer's disease [36], heart diseases [37] and prostatitis [38], determining the potential activity of drugs early in clinical development [39] and many more scenarios.

Hypoxia is another physiologically relevant characteristic of 3D microenvironments *in vivo* and *in vitro* [40]. In tissues, the concentration of oxygen is dependent on the balance between oxygen supplied and consumed. This balance is well controlled *in vivo* by evenly distributed capillary networks, which are lacking *in vitro*. Therefore the core of a homotypic 3D microtissue might become hypoxic as the size of the tissue increases. This event can cause induction of chemical signals (cytokines) from the cells for angiogenesis, which is similar to the way normal tissues respond to hypoxia where balanced signaling mechanisms lead to angioadaptation and vascular remodeling until the concentration of oxygen in tissue is back within its normal range [41]. Hypoxia-inducible transcription factors (HIFs) are the molecular mediators of physiological hypoxia and the cellular response. Under hypoxic conditions, HIF-1α and HIF-1β translocate to the nucleus [42], where their dimerization takes place and they subsequently bind to target gene motifs called hypoxia-responsive elements (HREs) resulting in altered gene expression [43]. HIF-1α seems to regulate the production of VEGF because its

suppression has been shown to cause transcriptional inhibition of VEGF [44] and reduced vascular density [45]. Hypoxic conditions have also been shown to trigger AP-1 [46] and NF-κB [47], which affects the production of a myriad of cytokines (see Table S1 in supplementary material online). The effect of hypoxia on cytokine production was further established by a study that showed higher levels of cytokines produced by hypoxic 3D co-cultures of CD34 and human umbilical vein endothelial cells (HUVEC) than normal cultures [48]. In another study, by Fischbach *et al.* [49], hypoxia was found to be associated with upregulation in cytokine secretion in 3D tumors. However, comparing monolayer cultures under reduced oxygen concentration with 3D cultures indicated that the sole contribution of hypoxia is comparably small and that the joint effect of hypoxia and the 3D microenvironment is necessary to elicit increased expression of cytokines, particularly IL-8 [49].

**Intercellular crosstalk in 3D culture: integrin–FN interaction**

Integrin α5β1 is the only integrin that naturally assembles FN into a matrix and this can lead to the formation of an endogenous matrix. FN is a multifunctional component of the extracellular matrix, which exists in a dimeric state, with the two chains attached through disulfide bonds at the C terminus [50]. Every FN chain has a single cell-binding domain having an arginine-glycine-aspartic acid (RGD) sequence to which α5β1 integrin specifically binds [51]. FN matrix assembly structural make-up includes the dimeric structure of FN, the N-terminal assembly domain and FN-binding sites in the first two type III repeats [52,53] and integrin binding to the RGD sequence in the cell-binding domain [54]. Interaction of integrin with FN promotes intermolecular association between the FN dimers, leading to the formation of fibrils, which further increases cell adhesion and cohesivity. This is consistent with the fact that monomers of

FN could not lead to aggregate formation [27] and this substantiates the notion that FN matrix assembly can support aggregate cohesivity by forming a scaffold or an organized 3D matrix, which leads to a functional linkage between cells. Using α5-, αV- and β1-integrin-blocking antibodies [28] caused a reduction in the amount of FN produced in fibroblast spheroids. This suggests that these integrins are involved in the expression of FN in spheroids but that the formation of tight compact spheroids is mediated through the α5β1 integrin only.

Integrin α4, α5 and β1 subunits are important for embryonic development *in vivo*, because their absence has been shown to be lethal to mouse embryos [55]. It has also been observed that a perfect correlation exists between the cohesion of the germ layers in amphibian gastrulae and the spatial position of these integrins [56]. Moreover, injecting RGD peptide into amphibian blastulae has been shown to disrupt cell interactions with FN causing blockage of gastrulation and preventing formation of FN fibril meshwork involved in migration [57]. If the interaction between integrin α5β1 and FN is indeed responsible for the cohesivity of tissues, particularly very early in development when embryos are essentially microtissue aggregate-like, then it is possible that this particular interaction also has the capacity to provide cohesive forces to cells within a microtissue *in vitro*.

Compaction mediated by FN has also been shown to be of importance in the later stages of development. A correlation has been found between FN production and pre-cartilage mesenchymal condensation during the development of wings and leg buds in chick embryos [58], with higher FN expression correlating with compaction of tissues. The interaction between integrin α5β1 and FN has also been shown to generate the tractional force that is essential for retraction of 3D FN–fibrin clot matrices [59]. This retraction process is crucial for early wound healing and tissue remodeling. It has been proposed that this intercellular cohesivity also

contributes to clot retraction, akin to the apparent retraction or compaction observed for CHO aggregates in response to increased concentrations of FN. Ergo, this cell–cell cohesivity produced by integrin engagement with FN is probably responsible for triggering the intracellular signaling pathways that lead to the difference in cytokine production seen in 2D monolayers and 3D microtissues. These pathways and their key molecular regulators have been elaborated in the following section. Evidence in support of involvement of integrin-mediated cell adhesion to FN in the production of cytokines has been provided by a study where microarray analysis done on myeloma cells growing on FN showed upregulation of many NF-κB-regulated genes [60]. NF-κB controls the expression of many growth factors and cytokines as discussed below. Some of the cytokines that were found to be upregulated, compared with control suspended cells, included TNFAIP2, CCL4, IL-6 and IL-8. Another study by Fischbach *et al.* [61] showed how transition from a monolayer to a 3D environment led to an increase in the production of IL-8 and VEGF by breast cancer, glioblastoma and oral carcinoma cells *in vitro* and *in vivo* in a SCID mouse model. The results from this study [61] suggest that the 3D microenvironment plays a vital part in regulating the secretion of IL-8 but that the combined effects of the 3D microenvironment, 3D cell morphology and 3D integrin (particularly α5β1) engagement are required to fully regulate the production of IL-8 by tumor cells. Interestingly, engagement of integrins in 2D and spread morphology of cells in a 3D space led to a higher IL-8 production than a conventional monolayer but less than 3D cultures, further emphasizing that spatial cues along with cell–ECM interactions and morphology of the cell regulate its cytokine secretion. Also, cell morphology is an often neglected but an important parameter because alterations in morphology cause actin cytoskeleton remodeling that can lead to differential NF-κB signaling [89] [s7]which controls cytokine production.

Integrin-mediated adhesion regulates many important intracellular signaling cascades. These pathways are most likely aberrantly regulated in 2D cultures because of the absence of an endogenous extracellular matrix assembly or cell–cell interactions. However, in a more physiologically relevant microenvironment, they can relay signals through a variety of adapter proteins that are localized at their cytoplasmic tails forming the focal adhesion complexes. Integrin signaling is mediated through focal adhesion kinase (FAK), Rous sarcoma oncogene cellular homolog (Src), adapter protein involved in oncogenesis (Shc) and growth factor receptor-bound protein 2 (Grb2) to downstream kinases. The Ras/Raf/ERK pathway is one of the most important pathways regulated by integrin-mediated adhesion. ERK activates several transcription factors in the nucleus such as NF-κB, AP-1, cAMP response element-binding protein (CREB) and E26 AMV virus oncogene cellular homolog (Ets-1) that control the production of cytokines and survival and growth factors important for the cell. Therefore, through proper activation of the ERK pathway, integrins can indirectly regulate the production of cytokines, provided they interact with a tissue-mimicking environment having optimum spatial, biophysical and biochemical cues.

**The link between the microenvironment and gene expression**

***Focal adhesion kinase***

FAK is a type of non-receptor tyrosine kinase that affects the dynamics of integrin-associated adhesion and the actin cytoskeleton that is coupled to it, through various molecular interactions. Being a component of focal adhesion scaffolding, it performs protein–protein interaction adaptor functions at the loci of cell adhesion to the ECM and also relays adhesion and growth-factor-

dependent cues into the cell body. Many reports have linked upregulated FAK expression with tumor growth [62,63]. The *FAK* gene locus at 8q23-q24 has also been shown to be a target of gene amplification during tumor progression [65]. [s8]However, as far as cytokine production is concerned, the gene is controlled by FAK through the Ras/Raf/ERK signaling pathway and activation of its downstream transcription factors NF-κB and AP-1. FAK signaling has been directly implicated in VEGF production by avascular tumors *in vivo* [66]. FAK expression and its catalytic activity as a result of Y925 phosphorylation promotes the ERK pathway, leading to an upregulation in VEGF expression resulting in tumor neovascularization without any significant alteration in cell proliferation or anchorage-independent survival. Inhibition of FAK activity by stable FAK C-terminal domain (FRNK) expression led to reduced secretion of VEGF compared with control in 4T1 breast carcinoma cells [66]. Cells expressing FRNK showed formation of small tumors that were avascular, without exhibiting differences in tumor-associated apoptosis. It was observed that FRNK led to the inhibition of a FAK/Grb2/MAPK signaling linkage controlling VEGF production. This finding was further supported by point-mutations that affected FAK catalytic activity or Y925 phosphorylation, disrupting the ability of FAK to promote ERK- and VEGF-associated tumor growth. Reduction in VEGF expression was also observed when FAK expression was inhibited in prostate, breast and neuroblastoma cells. Furthermore, a point mutation (FRNK S-1034) that inactivates FRNK by disrupting its co-localization with integrins leads to the restoration of FAK activity and VEGF production [67]. Another major factor that activates VEGF expression is the development of hypoxia within the core of the proliferating tumor [68]. Interestingly, hypoxia has also been found to increase FAK tyrosine phosphorylation and bolster the linkage between FAK and Grb2 in cardiac myocytes *in vitro* [69]. FAK overexpression has also been associated with neovascularization of the retina in

a mouse model of hypoxia-induced retinal angiogenesis [70]. Further evidence of FAK involvement in VEGF production is presented in the studies where overexpression of FAK in vascular endothelial cells led to angiogenesis in transgenic mice [71]. Additionally, FAK expression was found to be upregulated in angiogenic blood vessels within astrocytic-associated tumor stroma in comparison with normal brain endothelial cells [72]. Also, expression of FAK within endothelial cells is essential for vasculogenesis during development [73].

***The pathway to cytokine upregulation: Ras/Raf/ERK signaling and transcription factors***

The extracellular signals are relayed from the integrin via FAK and Src, leading to the activation of several intracellular signaling pathways, mainly the Ras/Raf/ERK pathway (Figure 3). In the context of cytokine production, Raf activity has been implicated in many studies. Transformation of hematopoietic cells by activated *Raf* genes has often been shown to result in the expression of granulocyte macrophage-colony stimulating factor (GM-CSF), which acts as an autocrine growth factor [74,75]. NIH-3T3 cells expressing activated Raf have shown increased secretion of heparin-binding epidermal growth factor (hbEGF) [76]. Kaposi's sarcoma transformed B cells that exhibit elevated expression of B-Raf also show an increased production of VEGF [77]. It has been shown recently that the infectivity of Kaposi's sarcoma virus is increased by B-Raf expression [78] and induction of VEGF production by B-Raf might be a mechanism for this elevation in viral infection [79]. Moreover, Raf activity leads to the subsequent activation of mitogen-activated protein kinase (MEK)1/ERK which activates downstream ERK which regulates the activation of transcription factors like NF-κB and AP-1 (Figure 3) that have been directly implicated in the expression of cytokines, mitogens and cell survival factors (see Table S1 in supplementary material online). The involvement of these transcription factors is discussed

in detail in the following subsection. The activity of Raf is regulated positively through phosphorylation on S residues in its catalytic domain. All three members of the Raf family (B-Raf, Raf-1 and A-Raf) have the capability to phosphorylate and activate MEK with different biochemical efficiencies [80]. This step is also adhesion dependent and is brought about by activation of endogenous p21-activated kinase (PAK) by small GTPase Rac induced by integrin-mediated adhesion to FN. B-Raf, which also directly activates MEK1 independently of Raf-1, is activated by integrin-bound focal adhesion complexes. However, Raf-1 can also activate NF-κB through a mechanism independent of MEK1/ERK by degradation of its inhibitor IκB via MEK kinase 1 (MEKK1) [81] (Figure 3).

ERK1 and ERK2 activities are regulated positively through phosphorylation mediated by MEK1 and MEK2 and can directly activate many transcription factors like Ets-1, AP-1 and c-Myc. ERKs can also regulate the phosphorylation and activation of the 90 kDa ribosomal S6 kinase (p90Rsk or RSK1), which in turn controls the activation of the transcription factor CREB [82]. Moreover, it has also been observed that p90Rsk can lead to the activation of NF-κB by phosphorylating its inhibitor IκBα at Ser32 causing its ubiquitination [83] (Figure 3). The localization of activated ERK is important in determining the cellular fate. To carry out the phosphorylation of its downstream transcription factors, ERK needs to be translocated from the cytoplasm to the nucleus. This is an adhesion-dependent step and is brought about by PAK activation by small GTPase Rac, induced by integrin-mediated adhesion to FN. In cells lacking adhesion cues (suspended cells), ERK activation can be achieved by overexpressing active Raf (22W Raf) or MEK (MEK1-ΔED) mutants. However, the signal is transmitted poorly to the nucleus because activated ERK cannot translocate to the nucleus and fully activate its downstream transcription factors [84]. Most of the cytokines and growth factors are the target

genes of NF-κB, AP-1 and Ets (see Table S1 in supplementary material online) which are in turn regulated by ERK or other components of the MAPK pathway (Raf and p90Rsk) and so proper control and activation of the pathway is essential for production and regulation of the cytokines [85].

***NF-κB***. [s9]NF-κB activation can be brought about by a variety of stimuli and, thus far, two NF-κB signaling mechanisms – classical and alternate – have been described [86]. The main NF-κB dimer that is activated through the classical pathway is p65:p50. Translocation of p65:p50 to the nucleus leads to the transcription of many proinflammatory targets, such as cytokines, chemokines, proangiogenic factors, adhesion molecules, antiapoptotic proteins and inducible enzymes. NF-κB activity can be regulated either in an ERK-independent fashion by Raf-1 via membrane shuttle kinase MEKK1 or in an ERK-dependent manner by RSK1 (as discussed in the previous section). The activation of both these pathways (Raf-1/MEKK1 or ERK/RSK) is dependent on integrin-mediated adhesion to the ECM and is regulated by the microenvironment and therefore requires physiologically relevant microenvironmental cues to be properly activated. NF-κB was found to be upregulated in hepatic stellate cells (HSCs) in 3D collagen I gel cultures [87] and in mammary tumor cell spheroids, where its activation was also responsible for resistance to apoptosis [88]. Many studies have also correlated NF-κB activation with cytokine production in different 3D cultures. In most of these studies, cytokine production in control 2D monolayer cultures was either lacking or at basal levels. Fibroblasts, when grown in spheroids, have shown an inverse correlation between NF-κB and IκB at a time point just before cytokine secretion [31]. The degradation of IκB leads to the activity of NF-κB in spheroids, whereas monolayer levels remained unchanged and the correlation was further substantiated

when the activity of NF-κB was found to be higher in 3D than monolayers at the same time points [31]. Also, changes in cell morphology can lead to production of cytokines because changes in the actin cytoskeleton lead to differential activation of NF-κB [89]. Such cytoskeletal changes are evident in transition from monolayer to a 3D culture platform [61]. Furthermore, it has been shown that FN is a potent activator of the NF-κB signaling pathway [90] and α5β1-integrin-mediated formation of FN endogenous matrix assembly leading to cellular cohesivity in 3D cultures as described earlier. Taken together, this information suggests the basis for upregulation of cytokine production in 3D cultures in comparison to 2D or monolayer cultures.

***AP-1***. The AP-1 transcription factor is not a solitary protein; it is instead made of various dimeric basic-region leucine zipper (bZIP) proteins that belong to the Jun, Fos, Maf and ATF [91] families. The activity of AP-1 is induced by various stimuli such as cytokines, growth factors, polypeptide hormones, neurotransmitters, bacterial and viral infections, cell–matrix interactions and many physical and chemical stresses. These stimuli lead to the activation of mitogen-activated protein kinase (MAPK) signaling cascades [92] that upregulate AP-1 activity through phosphorylation of distinct moieties. AP-1 activation controls the expression of several cytokines and CAMs. In monolayers, AP-1 activity might be impaired because the ERK pathway is differentially regulated owing to the lack of optimum microenvironmental factors. However, in 3D cultures, in the presence of a physiologically relevant tissue mimicking microenvironment, AP-1 activity leads to cytokine production, depending on the cell type (malignant versus primary) and/or scenario (tumor progression or wound healing). This hypothesis is substantiated by the fact that recently AP-1 was found to be activated in spheroids of different cell types culminating in cytokine production. All three cell types tested – transformed human embryonic

kidney (HEK293) [93], primary human foreskin fibroblasts (HFF-2) and carcinogenic glioblastoma (T98G) [94] – showed activation of different pathways leading to spheroid formation, quiescence and survival but the signals converged to the activation of AP-1 resulting in higher cytokine production than control monolayer cultures. This shows that response to microenvironmental cues can be cell-line-specific in terms of pathways but the final outcome (autocrine cytokine signaling and survival in this case) is dependent on the same transcription factor, further highlighting its importance. Furthermore, such autocrine and paracrine signaling is lacking in monolayer cultures owing to lack of proximity between cells and absence of cell–cell interactions and crosstalk.

***Ets***. Ets represents a family of transcription factors, including Ets-1, Ets-2, Elk-1, SAP1, SAP2, E1AF, PEA3, PU1, among others [95]. The Ets transcription factors regulate the expression of various other transcription factors like p53 [96], c-Fos [97] and NF-κB [98]. Ets proteins usually activate their target genes through association with other transcription factors. For instance, Ets-1 regulates the expression of GM-CSF in Jurkat T cells [99] and mast cells by coordinately regulating the GM-CSF promoter in partnership with NF-kB and/or AP-1 factors [100]. It has been shown that Ets-1 and Ets-2 induce the expression of the IL-5 promoter reporter gene construct in Jurkat T cells on stimulation with PMA (phorbol ester) and ionomycin [101]. Ets-1 also bound to an IL-5 promoter reporter gene when transfected into the mouse D10.G4.1 Th2 T cell clone, where it strongly cooperated with an adjacent AP-1 binding motif [102]. Also, co-transfection of Ets-1 and AP-1 (Fos/Jun) proteins into D10.G4.1 cells was able to induce expression of the endogenous IL-5 gene in the absence of exogenous activator molecules like PMA or cAMP that normally induce IL-5 expression in these cells [102]. This cooperative

activity is consistent with the production of cytokine in 3D cultures, where ERK cascade-controlled AP-1 and Ets activation is induced as a result of the presence of a physiologically relevant milieu and cellular crosstalk, synergistically driving cytokine production without the presence of any external induction molecule. Furthermore, Ets-1 binding sites have also been found in the promoters of many chemokines like CXCL4 (PF4) [103,104], CCL2 (MCP-1) [105] and CXCL8 (IL-8) [106], and Ets-1 knockout (Ets-1$^{-/-}$) or silencing (siRNA) has been shown to block the induction of these chemokines.

**Concluding remarks**

We have methodically built a case for the potential of cytokines as biomarkers for three-dimensionality or 3D cultures. The fact that cytokines are secreted in media makes their detection easier, especially in HTS readouts. Additionally, cytokines seem to be expressed in a wide range of cells from the four tissue types (muscle, connective, epithelial and nerve), which suggests their good potential for ubiquity as opposed to being cell- or tissue-specific. Also, their temporal expression suggests use of profiles as opposed to single-time-point measurements, increasing their robustness as biomarkers. For these reasons, cytokines are particularly attractive as biomarkers. As pointed out above, cytokines are broadly classified into seven subfamilies. In ongoing investigations in our lab (and possibly other laboratories), the question of which subfamily is most suitable to target for follow-up validation studies is being addressed.

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**Figure legends**

**Figure 1.** [s12]Schematic depiction of a total microenvironment (3D culture) supporting the formation of microtissue that exhibits 'complex' physiological relevance (CPR) or better emulation of the *in vivo* tissue functionality in a manner not possible in 2D cultures. The four factors shaping this microenvironment are: (i) chemical or biochemical composition; (ii) spatial (geometric 3D and topography); (iii) substrate biophysical properties; and (iv) temporal. Chemical cues can be subdivided into short-range (e.g. cell–cell) and long-range (e.g. diffusible species like nutrients and growth factors). Physical cues consist of shear as a result of fluid flow and/or physical properties of the material or scaffolding [10]. Spatial (a 3D space) and biochemical (protein coating) is of initial ($t_0$) importance, whereas there seems to be a lesser degree of dependence on biophysical cues [11]. As the time in culture increases ($t_1$), the cells produce an endogenous extracellular matrix (ECM), which has a physiologically relevant fibrillar conformation as opposed to exogenously supplied protein coating [125]. [s13]The cells send signals to neighboring cells for chemotaxis (e.g. cytokines), which is proposed herein for early biomarking of three-dimensionality as depicted in the intermediate circle. Once on the trajectory, the 3D microtissues should yield endpoint physiologically relevant outcomes (structural and/or functional) known *in vivo* but absent in 2D formats (e.g. microvilli-lined bile canaliculi and secretion of albumin for hepatic platforms) [125]. These outcomes can serve as the gold standard for formed microtissue emulation of its native counterpart. The microtissue can continue in a quasi-steady state until the size reaches a critical limit and oxygen and nutrient (biochemical factors) diffusion limitations lead to the initiation of a hypoxic phase, especially in constructs that cannot impose a physical constraint on the size of the microtissue [40].

**Figure 2.** HTS [s14]scaffold material discovery conceptual framework. **(a)** Chemical structure of pDTEc. **(b)** Chemical structure of and pDTOc. **(c)** Combinatorial fabrication of pPDTEc-pDTOc polymer blend scaffold library schematic. **(d)** Porogen-leached and freeze-dried sample of scaffold library in a 96-well plate. Adapted, with permission, [s15]from [7].

**Figure 3.** [s16]Extracellular signal-regulated kinase (ERK) and subsequent transcription factor activation via extracellular matrix (ECM)–integrin binding. Upstream events in the signaling cascade like integrin-mediated adhesion to ECM lead to GTP loading of Ras – in many ways like activation of Src and phosphorylation of Shc and Grb2. Transmission of the signal to Raf and subsequently to ERK kinase (MEK) and ERK is mitigated in suspended cells as there exists an evident anchorage-dependent step between Ras and Raf. A similar anchorage-dependent signal relay seems to be present between Raf and MEK. These two activation steps are regulated by integrin-dependent p21-activated kinases (PAKs). The activation of ERK can be achieved through heterologous expression of active upstream components like Raf (22W Raf) or MEK1 (MEK1-ΔED) in suspended cells but still the signal is transmitted poorly to the nucleus [84] because integrin activated Rac regulates ERK translocation to the nucleus along with controlling ERK activation through PAK. MEK activation is also controlled by B-Raf, which in turn is activated through engagement of focal adhesion kinase (FAK) with the actin cytoskeleton and subsequent formation of focal complexes. There is also a close interplay between the ERK pathway and nuclear factor (NF)-κB, with Rsk [83] and Raf [81] regulating its activation. In the nucleus, ERK is involved in the activation of all the three subclasses of the Ets family of transcription factors and formation of the ternary nucleoprotein complex between TCFs (Elk-1, Net and Sap-1) and serum response factor (SRF) over the serum response element (SRE) of the promoter. Activation of RSK in the cytoplasm and translocation to the nucleus further activates

cAMP response element-binding protein (CREB) [85]. Abbreviations: EGFR, epidermal growth factor receptor; Src, Rous sarcoma oncogene cellular homolog; Shc, adapter protein involved in oncogenesis; GRB2, growth factor receptor-bound protein 2; Crk/C3G, CT10 sarcoma oncogene cellular homolog/guanine nucleotide-releasing factor 2; Rap1, repressor activator protein 1; MEK, MAPK/ERK kinase; Rsk, ribosomal protein S6 kinase; TNFR2, tumor necrosis factor receptor 2; TIM, T cell immunoglobulin- and mucin-domain-containing molecule; RIP, receptor-interacting protein; TRAF1, TNF receptor-associated factor 1; TRAF2, TNF receptor-associated factor 2; cIAP, cellular inhibitor of apoptosis protein; IKK, IκB kinase; Ub, ubiquitin, IκB, inhibitor of NF-κB; MEKK1, MAPK/ERK kinase kinase 1; NIK, NF-κB-inducing kinase; MSK1, mitogen and stress activated kinase 1; ATF, activating transcription factor; CBP, CREB binding protein; SRF, serum response factor; ERF, ETS2 repressor factor; AP-1, activator protein 1; Ets, E26 AMV virus oncogene cellular homolog; Elk, Ets-like transcription factor.

Amish Asthana

Dr Amish Asthana is a Post-Doctoral Research Associate in the Cellular Bioengineering Laboratory at the University of Georgia. His main research focus is developing physiologically relevant 3D cell-based assays for drug discovery applications. He has authored multiple peer-reviewed publications and has mentored several graduate and undergraduate students. He has extensive hands-on experience in fabricating polymer and other scaffold types, characterization of hepatic and neural cells that are cultured on them, cellular and molecular biology techniques and biomanufacturing. He obtained his BTech in Biotecnology from Vellore Institute of Technology, India, and his PhD in Biological Engineering from UGA.

**William S. Kisaalita**

Dr William S. Kisaalita is a Distinguished Faculty Scholar and a Professor at the College of Engineering at the University of Georgia. He received his PhD in chemical engineering from the University of British Columbia. He directs the Cellular Bioengineering Laboratory. The focus in the lab is on the development of high-throughput, 3D cell-based assays with applications in drug discovery. He is the author of a reference book: *3-D Cell-based Biosensors in Drug Discovery Programs*, published by Taylor and Francis. Apart from 3D tissue engineering, Dr Kisaalita has interest in the development and commercialization of income-increasing or labor-reduction technologies for low-resource settings.

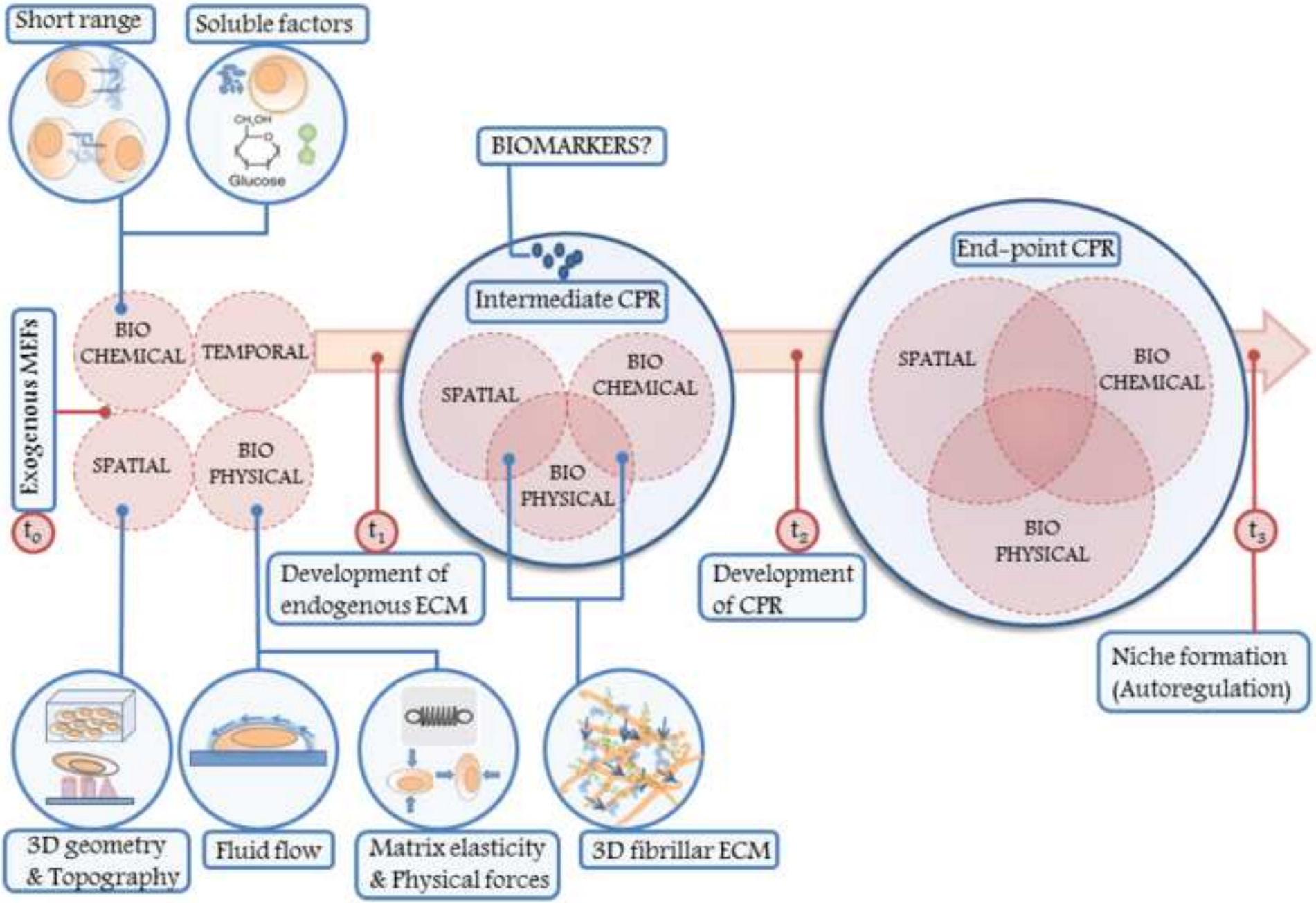
**Table 1. Transcriptomic–proteomic upregulation of cytokines in 3D cultures**

| Cell line | Cell type | 3D scaffold | Cytokines[a] | Refs |
|---|---|---|---|---|
| Fibroblasts | Primary (biopsy) | Collagen matrix | **IL-6**[s17] | [107] |
| C6 | Rat glial tumor cell line | Spheroids | VEGF | [108] |
| MCAS, SKOV/MG | Ovarian cancer cell lines | Spheroids by liquid overlay | VEGF | [109] |
| IMR-90 | Human fetal lung fibroblasts | Collagen – GAG matrix | IL-8, CXCL1, CXCL2, CXCL3, CXCL5, VEGF, LIF | [110] |
| NA8 | Melanoma | Spheroids on pHEMA plates | **CXCL1, IL-8, MIP-3a,** angiopoetin like 4, CXCL2,3 | [111] |
| MG-63, SaOS-2 | Human osteosarcoma | Si-HPMC polymer hydrogel | IL-6, GM-CSF | [112] |
| R1 | Murine ES cells | Cytomatrix RW-spinner culture | BMP-4, IGF2 | [113] |
| L1236 | Hodgkin-lymphoma-derived cell line | RADA-oligopeptide matrix | CCL4, CCL5, CCL17, CCL22, IL-13, TNF, TNFS2, TNFS7, INHBA | [114] |
| NA8 | Melanoma | Spheroids on pHEMA plates | IL-8, VEGF, ANGPTL4 | [115] |
| PDAC | Pluripotent progenitor cell | pECM | **IL-6, IL-8, MCP-1** | [116] |
| HFSF-132 | Human foreskin fibroblasts | Spheroids | **IL-1β, IL-6, IL-8, IL-11, GM-CSF, LIF** | [117] |
| HFSF, CRL-2088<br>HES<br>HAL<br>MRC-5 | Human foreskin fibroblasts<br>Embryonic skin fibroblasts<br>Adult lung fibroblasts<br>Embryonic lung fibroblasts | Spheroids on agarose plates | **CCL2-5, CXCL1-3, 8**<br>**CXCL8**<br>**CXCL8**<br>**CXCL8** | [31] |
| OSCC3<br>U87<br>MDA-MB231 | Oral squamous cell carcinoma<br>Glioblastoma<br>Breast cancer | PLG, RGD alginate, Matrigel® | **IL-8, VEGF** | [39] |
| BMSC | Bone-marrow-derived mesenchymal stroma cells | Spheroids on agarose plates | CXCL12, Wnt5a, KITLG | [118] |
| hMSC | Mesenchymal stem cells | Spheroids | IL-24, LIF, Stanniocalcin-1 | [119] |
| U251 | Glioma cell line | Collagen-coated PLGA 3D scaffolds | **VEGF, bFGF** | [120] |
| PLC, HepG2 | Hepatocellular carcinoma | Chitosan-alginate | **VEGF, bFGF, IL-8** | [121] |
| OSCC3 | Oral squamous cell carcinoma | Alginate | **VEGF, IL-8** | [122] |

| | | | | |
|---|---|---|---|---|
| U87 | Glioblastoma | | | |
| 143.98.2 | Osteosarcoma | Silk fibroin scaffold | VEGF, IL-8 | [123] |
| MDA-MB-231 | Breast cancer | 3D fibroin scaffold | **VEGF, IL-8** | [124] |

[a]Bold indicates protein results, other results are only transcriptomic.

**Figure 1**


Short range
Soluble factors
Glucose
BIOMARKERS?
Intermediate CPR
End-point CPR
Exogenous MEFs
BIO CHEMICAL
TEMPORAL
SPATIAL
BIO PHYSICAL
SPATIAL
BIO CHEMICAL
BIO PHYSICAL
SPATIAL
BIO CHEMICAL
BIO PHYSICAL
$t_0$
$t_1$
$t_2$
$t_3$
Development of endogenous ECM
Development of CPR
Niche formation (Autoregulation)
3D geometry & Topography
Fluid flow
Matrix elasticity & Physical forces
3D fibrillar ECM

# Figure-2 (Kisaalita)

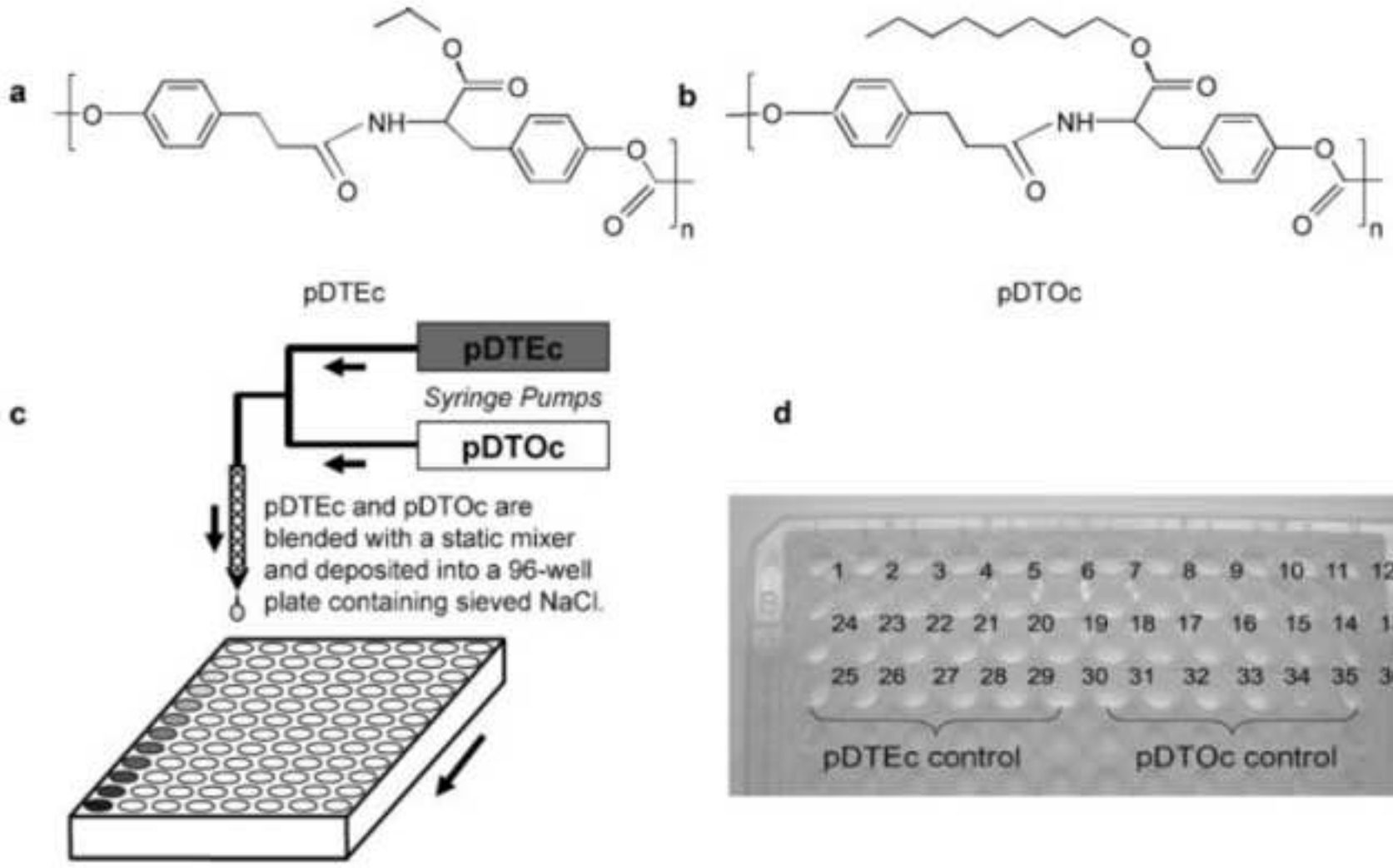
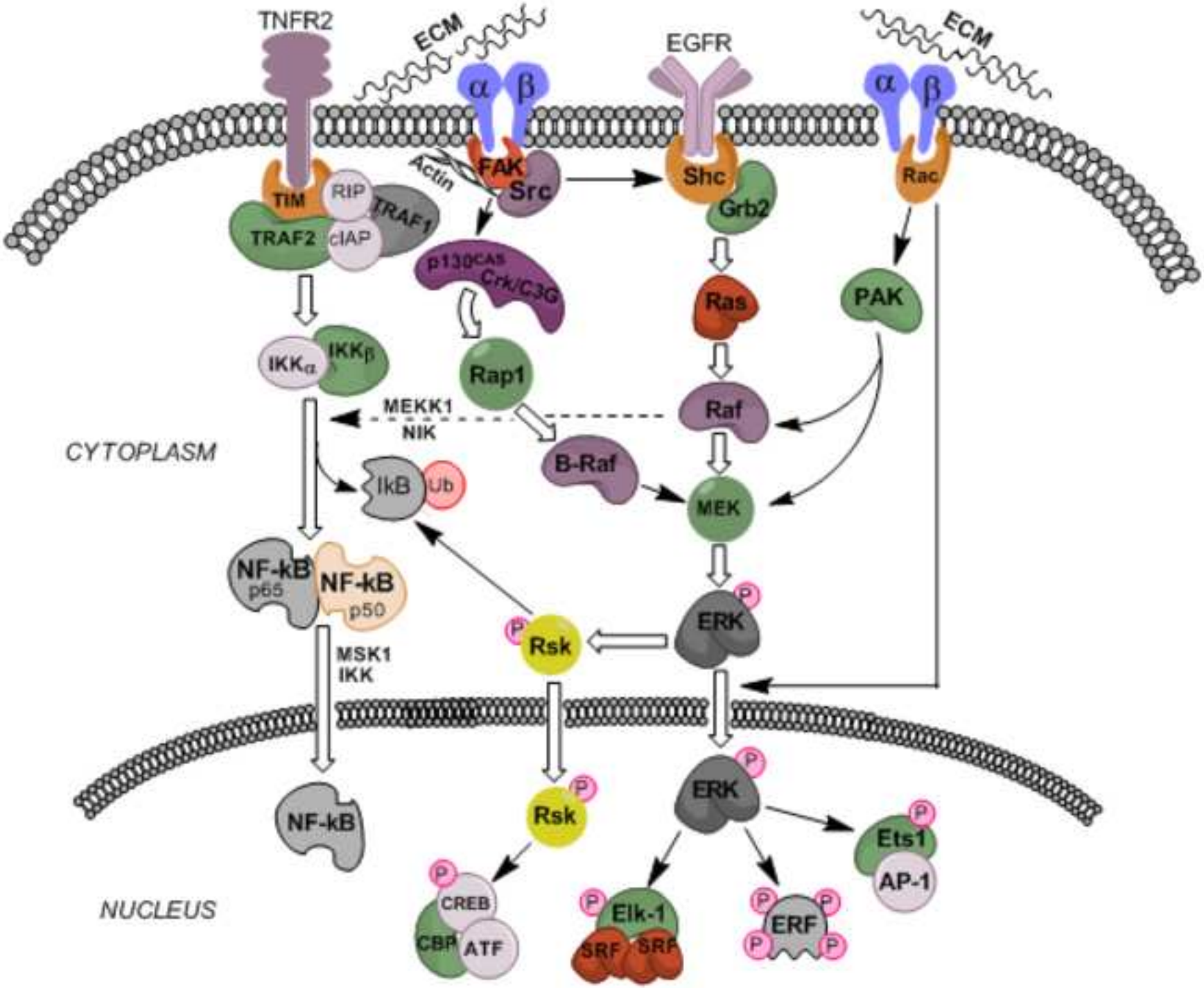


**Figure 3**


TNFR2
ECM
EGFR
ECM
α
β
α
β
FAK
Src
Actin
Shc
Grb2
Rac
TIM
RIP
TRAF1
TRAF2
cIAP
p130CAS
Crk/C3G
Ras
PAK
IKKα
IKKβ
Rap1
MEKK1
NIK
Raf
CYTOPLASM
B-Raf
IkB
Ub
MEK
NF-kB
p65
NF-kB
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Ets1
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CREB
CBP
ATF
Elk-1
SRF
SRF
ERF
NUCLEUS