Molecular Crowding – (in Cell Culture)

Cedric Badowski, Aneesa Iskander, Diana Gaspar, Dimitrios I. Zeugolis, and Michael Raghunath

Contents

Abstract

Macromolecular crowding (MMC) is an intrinsic and ubiquitous feature in biological cells. We find MMC in the first bacterial cell and see it culminating in the intricate extracellular matrix (ECM) that evolved in multicellular organisms. Research work on MMC started with the observation that biological cellular

C. Badowski
Institute of Medical Biology, A*STAR, Singapore, Singapore
e-mail: cedricbadowski@gmail.com

A. Iskander · M. Raghunath (✉)
Institute for Chemistry and Biotechnology (ICBT), Department Life Sciences and Facility Management, ZHAW Zurich University of Applied Sciences, Wädenswil, Switzerland
e-mail: e0011348@u.nus.edu; ragh@zhaw.ch

D. Gaspar · D. I. Zeugolis
Regenerative, Modular and Developmental Engineering Laboratory (REMODEL) and Science Foundation Ireland (SFI) Centre for Research in Medical Devices (CÚRAM), Biomedical Sciences Building, National University of Ireland Galway, Galway, Ireland
e-mail: DIANA.GASPAR@nuigalway.ie; Dimitrios.Zeugolis@nuigalway.ie

J. M. Gimble et al. (eds.), *Cell Engineering and Regeneration*, Reference Series in Biomedical Engineering, https://doi.org/10.1007/978-3-319-08831-0_50

systems are crammed with macromolecules. The interior of cells is teeming with enzymes, transport systems, and nucleotide assemblies. In addition, eukaryotic cells possess a three-layered cytoskeleton adding confinement to an already packed cytoplasm. Likewise, the extracellular space of multicellular organisms comprises an ECM consisting of fibrillar proteins, such as collagen or elastin, surrounded by an amorphous gel-like ground substance glycoproteins and proteoglycans and their hydration shells. Together, they provide mechanical resilience to the tissues of vertebrates while forming a crowded and structural microenvironment that in turn creates confinement for other macromolecules. Surprisingly, most biochemical and cell culture experiments are still done in non-crowded, highly aqueous solutions. Here, we shall discuss the shortcomings of contemporary cell culture and emphasize the benefits of applying MMC to cell culture models of tissues. MMC can be achieved by adding water-soluble macromolecules to the culture medium. Not only is this technically feasible, it also moves in vitro biology toward a higher physiological level, allowing the design of more meaningful cell-based assays and enabling tissue engineering of matured and physiologically relevant tissue-like assemblies.

1 Introduction

1.1 Shortcomings of Contemporary Cell Culture

Cell culture is a system that seeks to multiply cells extracted from living tissues in order to study them or to build new cell communities in vitro. So, first and foremost, the extracted cells need to stay alive. Cells come from a tissue context comprising neighboring cells and their microenvironment, the extracellular matrix (ECM). Cells are removed abruptly out of their optimal and complex tissue context (Cigognini et al. 2013) and are seeded usually on onto polystyrene, glass, or other surfaces. Robbed of their niche, cells must settle on a foreign surface, where they are expected to adhere, spread, and then divide by mitosis. At this point, it might be important to note that animal cells adhere to the proteins attached to the plastic dishes rather than the plastic itself. Indeed, plastic surfaces are treated to achieve fast binding of adhesive proteins, usually from serum, that mediate the actual cell adherence (Ramsey et al. 1984). Typical mediator proteins from serum are fibronectin and vitronectin, as they engage integrin receptors that all animal cells express on their surface (Hayman et al. 1985). Fibronectin is both an ECM and a serum protein. Serum fibronectin is a circulating biosynthetic product of the liver of the serum donor, while cellular fibronectin is made by the adhering cells (the vast majority of anchorage-dependent cells, if not all, are capable of producing one form of fibronectin or another) (Stenman and Vaheri 1978; Owens and Cimino 1982). As cells adhere to the serum proteins, they will add their own synthesized fibronectin to this. For example, human cells grown in the presence of fetal bovine serum will form a hybrid matrix of bovine and human fibronectin. Thus, the cells coat foreign surfaces with their own adhesive matrix proteins to facilitate their own anchorage. Obviously,

in vitro animal culture is a highly artificial system designed for optimal multiplication of cells, convenience, visual inspection, and optical studies of cell cultures. Getting cells to survive outside a tissue context is rather challenging, and decades of research and development have gone into the formulations of culture media that are commercially available to date. However, convenience issues seem to be getting in the way of redesigning cell culture despite scientific evidence that current cell culture systems are deficient on several levels. We will review these shortcomings only briefly (for more detailed reviews, please see Chen et al. 2011; Park et al. 2015), but it is important to be aware of them. Some of these shortcomings are not easily remedied, while others can be, as we will demonstrate in this chapter.

For a start, let us consider the cell attachment surface. Current surfaces are made of glass (still the best for optical imaging and short-term culture) (Mather and Roberts 1998), polystyrene (Curtis et al. 1983), and polycyclic olefins (Niles and Coassin 2008). These surfaces offer no intrinsic purchase for anchorage-dependent cells, other than perhaps strong electrostatic interactions with the cell membrane. Most of the 213 cell types of the human body are anchorage-dependent and would not thrive in suspension, unless they are transformed or cancer cells (Benecke et al. 1978). Therefore, surfaces are treated to make them negatively charged and hydrophilic so that polar regions of the proteins can stick to them and, in turn, mediate cell attachment via presentation of peptide sequences to cell receptors. Such well-described sequences are the RGD motifs that engage integrins (Ruoslahti 1996). Without the presence of those "sticky" proteins, cells will often not attach to plastic, as the experience of seeding cells in the absence of serum teaches us (Barnes and Sato 1980). Classical adhesion-mediating proteins are ECM proteins, which are secreted and immobilized outside of cells as supramolecular assemblies (Grinnell and Feld 1979). Cells have transmembrane receptors that bind to these ECM assemblies, and therefore, commercial ECM extracts or synthetic ECM-derived peptides contain integrin recognition sequences (e.g., nectins, laminins) (Aplin et al. 1998; Lutolf and Hubbell 2005). These peptides are used to coat cell culture plastic or come pre-coupled to plastic. In this regard, the current concept of "plastic adherence" for the isolation and characterization of mesenchymal stromal cells as a distinguishing criterion from hematopoietic progenitor cells from the bone marrow (Dominici et al. 2006), is quite intriguing. In reality, this is a selection of serum-protein-adherent cells or of cells particularly attracted to certain plastic surface charges. Emulating this on a more synthetic basis are peptides derived from the nectin family of proteins that are directly coupled to plasticware. One might wonder how different batches and qualities of serum or peptide coatings might decide over the very first selection of mesenchymal progenitor cells for subsequent expansion and later functionality and therapeutic application for tissue repair. Research in this direction might uncover interesting findings on the natural history of cultured mesenchymal stem cells.

This brings us to the missing microenvironment. Cells seeded onto culture vessels do not find an intricate ECM to settle in. No member of the family of collagens, laminins, nectins, microfibrillar proteins, and proteoglycans is present to welcome freshly seeded cells and help them anchor. Moreover, cell fate is highly dependent on

the surrounding microenvironment. Indeed, a particular set of ECM proteins could constitute a specific niche that would, for example, maintain stem cells, while a different ECM composition could favor differentiation into other cell types. The ECM is even more complex than it seems, since various matrix proteins can also bind different growth factors that promote cell growth and/or differentiation (Chen et al. 2007; Lin and Bissell 1993). To compensate for this lack of ECM diversity, artificial matrices meant to emulate extracellular matrix are continuously developed and published in biomaterials journals with the intention to supersede nature (Lutolf and Hubbell 2005; Kyburz and Anseth 2015). However, most coating materials commercially available for plastic ware are mostly ECM extracts and polymers of animal origin, either cell lines or tissue extracts. These products do not come cheap and, in many cases, would face regulatory hurdles for cell therapeutic intentions. Conceptually, these products reflect a disregard of the fact that the microenvironments the cells were harvested from was originally made by these very cells. *Obviously, freshly seeded cells are deprived of a fitting microenvironment to start with*; *however*, *we also need to understand that they are hobbled by the culture conditions in the first place*.

Culture media as such constitute another level of challenge in animal cell culture models. Although the development of culture medium spans several decades, it has remained essentially the same system: cells are fully immersed in a seawater equivalent supplemented with carbon sources (e.g., glucose, pyruvate, glutamine, and other amino acids), vitamins, varying micronutrients, and a carbon dioxide-based buffer system (unless HEPES is being used) (Bettger and McKeehan 1986; Eagle 1955). So-called high-glucose media are on the market, because they allegedly promote cell proliferation. It might be worthwhile to consider that high-glucose medium in essence reflects a diabetic condition, and therefore uncontrolled non-enzymatic glycation of cell cultures might occur in the long run and not reflect physiology (Brownlee 2001; Ahmed 2005).

The focus of this book chapter is that, except for the urothelium of the urinary bladder, the inner lining of blood vessels, and perhaps the synovia of joints, cells in vivo are not exposed to large amounts of water, unless in a pathological situation like an edema. Despite all the additives mentioned above, most culture media cannot sustain viability of cultured cells, at least not at the desired proliferation rates. Therefore, an enigmatic component is added in the range of 0.5–20% v/v – fetal bovine serum (FBS) (Van der Valk et al. 2010; Arigony et al. 2013; Honn et al. 1975). FBS contains an array of hormones and stimulatory factors (Zheng et al. 2006), along with the abovementioned adhesive components: fibronectin and vitronectin. The global supply of FBS is limited; it comes from different countries with considerable batch to batch variations, leading to different proliferation and functionalities of cells cultured in those respective serum batches (Gstraunthaler et al. 2013). While endothelial cells might be continuously exposed to serum (plasma) proteins in some way, most other cell types might never see serum components in their entire life span, unless under pathological circumstances, such as leaky vasculature during inflammation or a wound. Current discussions to remove fetal bovine serum from culture media pivot therefore on reproducibility, concerns

on transfer of xenogeneic pathogens and immunology, as well as issues of animal welfare related to the harvest of the biological product (Van der Valk et al. 2004). However, an often lackluster performance of cells in serum-free preparations in combination with high costs has prevented a deeper penetration of serum-free culture media at present in research laboratories.

Growing cells in the classical incubator is performed in the dark, at a humid atmosphere of 5% carbon dioxide, atmospheric oxygen (20%), and at 37 °C. Firstly, the inside of a dark incubator might not provide sufficient electromagnetic stimulation (light) for cell types harvested from the body surface (corneal epithelium, dermal and epidermal cells, such as keratinocytes and melanocytes). More importantly, oxygen levels in arterial blood range from 9.5% to 13% and drop, in actual tissues, to as low as 0.5% (Mohyeldin et al. 2010). Atmospheric CO_2 is 0.04% but reaches 4.6% and 6% in tissues (Hoffman et al. 1998; Bolevich et al. 2016). It follows that cells are routinely grown in hyperoxic and thus stressful conditions, possibly leading to faster aging of cell cultures or cell lines that genetically drift due to the incorporation of mutations associated with reactive oxygen species (ROS). This hyperoxic environment may also affect cell fate since numerous stress-related pathways are involved in cell differentiation, notably by activating stress-activated protein kinases such P38 and JNK kinases (Lee and Choi 2003). Temperature wise, 37 °C might be ever only reached in the liver bed. Elsewhere, temperatures are typically lower by 2–3 °C (Werner and Buse 1988; Birnie and Grayson 1952). Although shear forces will be present in bioreactors with flow and cell cultures on microcarriers in suspensions that are continuously stirred (Ratcliffe and Niklason 2002), the absence of mechanical stimulation in conventional cell culture must also be mentioned here. Reintroducing macromolecular crowdedness into culture media, by ways of adding macromolecules, could not only remedy the wateriness of the culture medium but also solve the issue of insufficient microenvironment formation.

2 The Long and the Short of Macromolecular Crowding

The first application of macromolecular crowding in cell culture goes back 30 years. Firstly polyethylene glycol (4 kDa, 5% w/v), polyvinylpyrrolidone (30–40 kDa, 6.5% w/v), and dextran T-40 (40 kDa, 5% w/v) (Bateman et al. 1986) and later dextran sulfate (500 kDa, 0.01% w/v) (Bateman and Golub 1990) were shown to increase the conversion of procollagen to collagen. The authors showed an increased deposition of collagen in the ECM by fibroblasts in order to highlight the activity of bone morphogenetic protein 1, then known as procollagen C proteinase (Bateman et al. 1986). Thus, a method had been described to increase collagen deposition in vitro. It was also used to study collagen deposition by fibroblasts from a patient with osteogenesis imperfecta (Cetta et al. 1993), and in a cell-free system, dextran sulfate or polyethylene glycol was shown to speed up the activity of the same enzyme purified from chick embryo tendons (Hojima et al. 1994). In these publications, the terms “excluded volume” and “macromolecular crowding” were used, respectively, but the mechanism leading to an increased procollagen cleavage and collagen

deposition remained unclear; a protein precipitation and aggregation step of some kind were discussed at that time. In the last 10 years, we and others have been able to gain more mechanistic insights into the effects of MMC in cell culture.

All considerations of macromolecular crowding start with the notion that the interior of a cell is crammed with macromolecules. In addition, eukaryotic cells contain a cytoskeletal meshwork that adds confinement to this macromolecular system. These considerations have been reflected in numerous review articles (Minton 2001; Ellis 2001). But when we take a closer look at the extracellular space in multicellular organisms, we find a comparable situation: the immediate vicinity of cells is also crammed with macromolecules, many of the immobilized, thereby adding confinement to the microenvironment. Therefore, any molecule secreted into the extracellular space will encounter a crowded environment in vivo.

Another important point to consider is that, in chemical reactions, reactants are modeled as point masses. This means that the actual sizes of the reaction partners are negligible and are therefore indeed neglected. In biochemical reactions, however, size does matter. In comparison with their respective substrate, enzymes can be huge, for example, glucose (180 Da) and glucose oxidase (160 kDa) (Woolridge et al. 1986), or are dwarfed by it like DNA polymerases working on a DNA strand, or exhibit similar masses such as bone morphogenetic protein 1 (88 kDa) (Lee et al. 1997) and procollagen (100 kDa) (Bornstein 1974). This means that the size of a macromolecular crowder might matter substantially with regard to the sizes of the molecules partaking in a biochemical reaction that are to be modulated by MMC.

3 Paradigm Shift: Make Your Own Matrix Scaffold In Vitro

The ECM of tissues is composed of fibrillar proteins and a gel-like ground substance made of dozens of glycoproteins and proteoglycans that are secreted by and immobilized around matrix-producing cells (Theocharis et al. 2016). It is important to distinguish between the biosynthesis of ECM molecules, their secretion, and their extracellular assembly into water-insoluble structures – the *deposited* ECM (Mouw et al. 2014). Clearly, secretion does not necessarily follow biosynthesis, and deposition does not necessarily follow secretion.

The biosynthesis of ECM molecules depends on signals that are received by the cell, such as growth factors or breakdown products of ECM. The secretion of a variety of collagens, however, is greatly aided by the presence of vitamin C (Murad et al. 1981). The extracellular assembly that follows successful secretion is a supramolecular aggregation of partners forming polymeric fibrils, or mesh-like structures. The basic polymeric structures are represented by fibronectin, collagens, laminins, and elastic microfibrils (Theocharis et al. 2016). Some collagens are forming fibrils, such as types I, II, and VII, while other collagens use these already formed fibrils as assembly points without forming fiber systems, such as type IX and XI collagens (Gelse et al. 2003). In analogy, elastin binds to fibrillin-containing microfibrils. Others collagens like collagens III and V mix to form heterotypic fibrils with collagen I and form the bulk of the connective tissue of the dermis (Birk et al.

1990; Fleischmajer et al. 1990). These biopolymeric assemblies represent molecular landing sites for a variety of ligands that in turn fasten growth factors to the ECM. Examples here are latent transforming growth factor beta-binding protein (LTBP) binding to fibronectin or elastic microfibrils and capturing latent TGFbeta and negatively charged proteoglycans associated with fibrillary structures that capture positively charged growth factors, like FGF and VEGF. Finally, enzymatic and nonenzymatic modifications of these supramolecular assemblies lead to the maturation of the built cell microenvironment. This includes later remodeling, involving breaking down structures while making way for new ones and changes in shape, structure, and composition of this ECM (Royce and Steinmann 2003).

Earlier work suggests that macromolecular crowding can aid in enzymatic reactions by stabilizing complexes of enzyme and substrate, thus increasing enzymatic activities (Zhou et al. 2008). Interestingly, additional effects are seen through the deposition, stabilization, and remodeling of ECM and are dependent on a variety of enzymes (Fig. 1). To select a few, the intracellular posttranslational modification of collagen that is crucial to its thermo-stabilization and therefore successful release into the extracellular space is catalyzed by prolyl hydroxylase (Notbohm et al. 1992). The extracellular proteolytic conversion of procollagen to collagen is controlled by bone morphogenetic protein 1 (also known as procollagen C proteinase) activity (Fig. 1). The extracellular covalent crosslinking of collagens (and elastin) is effected by the copper-dependent enzyme lysyl oxidase and the calcium-dependent transglutaminase 2 (Wang and Griffin 2012). Remodeling, removing, and reshuffling of the ECM are performed by matrix metalloproteinases and other proteolytic enzymes (Bonnans et al. 2014). The activation of these ECM-modifying enzymes sometimes requires their own cleavage by proteolytic enzymes. Therefore, multiple potential targets for MMC with regard to enzyme-mediated modifications in the extracellular space arise (for comprehensive overview, see Royce and Steinmann 2003).

The first ECM-relevant observation with macromolecular crowding focused on the procollagen-converting enzyme bone morphogenetic protein 1/procollagen C proteinase. Macromolecular crowding induces faster conversion of procollagen to collagen, leading to stronger presence of cleaved collagen in the pericellular matrix and a correspondingly weaker presence of uncleaved procollagen in the culture medium (Bateman et al. 1986; Bateman and Golub 1990). In a cell-free system, the proteolytic activity of procollagen C proteinase on procollagen was found to be accelerated under MMC (Hojima et al. 1994). The addition of crowders to the culture medium was also shown to increase the conversion of procollagen to collagen in cell culture; immunochemical analysis demonstrated a substantial increase of the clipped-off collagen I C-propeptide trimer under crowding (Chen et al. 2011). Moreover, the authors demonstrated that procollagen C proteinase enhancer (PCPE), an allosteric regulator of procollagen C proteinase, was also proteolytically converted into an active enhancer of procollagen C proteinase activity and a smaller fragment that is speculated to have anti-collagenolytic properties, similar to tissue inhibitors of metalloproteinases (TIMP) (Mott et al. 2000). Interestingly, PCPE also activates the crosslinking enzyme lysyl oxidase (Trackman 2005). Our work demonstrated that MMC accelerated more than one

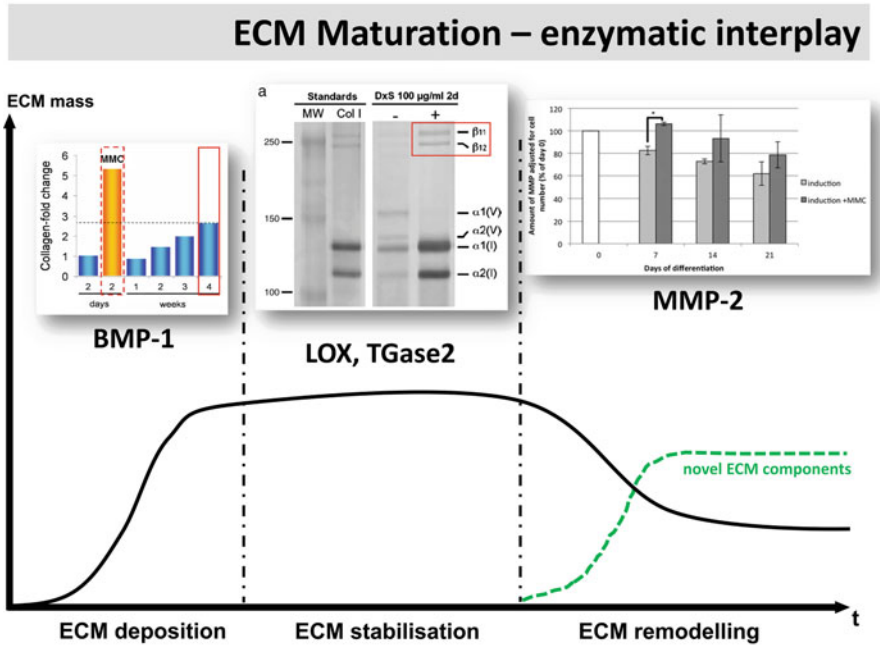

Fig. 1 Enzymatic key activities in the formation and maturation of ECM that are targets of MMC. Left: extracellular collagen deposition is boosted by accelerating bone morphogenetic protein 1 (*BMP1*), also known as procollagen C proteinase. The enzymatic activity shortens procollagen to collagen triple helices by removing globular C-termini. This trimming process now allows the assembly of collagen molecules to form insoluble fiber systems. This enzymatic process is rate-limiting for collagen deposition and slow in standard culture (blue columns). Therefore, the acceleration of this step via MMC (orange column) represents a substantial advantage in cellular microenvironment formation. (Reprinted from Lareu et al. 2007b, with permission from the publisher, Elsevier Center: Enzymes play a crucial role in the stabilization of deposited ECM). Shown here are high molecular weight double bands (red box) representing collagen alpha chains that have been covalently crosslinked by lysyl oxidase (*LOX*) activity. (Reprinted from Chen et al. 2011, with permission from Elsevier). Right: the remodeling of ECM involves the proteolytic removal of already deposited matrix components and often is accompanied by the deposition of new matrix. Therefore, not only the quantity but also the composition of the ECM will change as some ECM components will recede while others emerge. Here, matrix metalloproteinase 2 (*MMP2*) has been demonstrated via zymography to be strongly associated with matrix during adipogenesis under MMC. (Reprinted from Ang et al. 2014 with permission from Mary Ann Liebert Inc.)

converting enzyme in a cascade process. We also speculated that the binding of PCPE to procollagen C proteinase would be faster and tighter under crowding as well, which would have a tremendous effect, as PCPE is known to increase the catalytic activity of procollagen C proteinase by one order of magnitude (Lareu et al. 2007a) (Fig. 1).

Once procollagen is cleaved to collagen, the collagen molecules assemble as trimers in an orderly fashion, first forming nuclei and then extending and elongating into microfibrils that grow in length and girth. In parallel, crosslinking enzymes

stabilize these meshworks and assist to immobilize them and protect them against proteolytic attack (Bornstein 1974). Lysyl oxidase crosslinks have especially been described and investigated extensively in various connective tissues ranging from tendon, skin, and bone to cornea and blood vessels (Kielty and Grant 2003). Transglutaminase 2 also plays a role in covalently crosslinking collagen assemblies (Wang and Griffin 2012; Raghunath et al. 1999; Raghunath et al. 1996; Zeugolis et al. 2010) as well as affixing ligands to ECM assemblies such as latent transforming growth factor binding proteins (Raghunath et al. 1998; Taipale et al. 1996) (Fig. 1).

However, ECM also requires remodeling, when tissue changes arise. Obviously, growth of a tissue and repair processes after injury require the addition and the rearrangement of the ECM. One classic example is the cascade of events that occurs after the fracture of a bone (Alford and Hankenson 2006; Damsky 1999). Firstly, the adjacent ends of the fractured bone will be partially resorbed via decalcification and removal of collagen fibers, and then a collagenous soft tissue occurs between the borders of the fracture, the callus, which then finally gets fully calcified. Damaged and denatured ECM will be proteolytically removed, and new ECM needs to be deposited and welded to pre-existing structures. In this case, the connective tissue is replaced with a similar connective tissue. Cell differentiation processes also require remodeling not only in terms of shape but also in terms of composition. The formation of adipose tissue, for example, requires a drastic change of progenitor cells from spindle shaped to spherical as demanded by the intracellular accumulation of lipid droplets (Nakajima et al. 1998; Gregoire et al. 1998). In consequence, the ECM, having to accommodate different cell shapes, will switch from a collagen I- and fibronectin-rich ECM to an ECM dominated by collagen IV, invoking the reduction of fibronectin. This sequence of events in adipogenesis involving proteolytic remodeling of the ECM was first proposed in culture in murine pre-adipocyte 3T3-L1 cells (Lilla et al. 2002; Selvarajan et al. 2001) and was confirmed later in human cells by differentiating human bone-derived mesenchymal stromal cells under MMC in vitro (Ang et al. 2014). Here, proteolytic enzymes that are specific to ECM components and assembly thereof come into play. We have shown that MMP-2 is more closely associated with the ECM under crowding conditions and that MMP activity can be accelerated under crowding conditions in a cell-free system (Ang et al. 2014) (Fig. 1).

4 Not to Be Overlooked: Collagens and Ascorbic Acid

We have shown that MMC greatly accelerates enzymatic processes that facilitate collagen matrix formation and deposition, which are normally tardy in standard cell culture. However, it cannot be stressed enough that any of these enzymes can only work with ECM components that are available in the pericellular space. This is where ascorbic acid (Vitamin C) comes in, as it is responsible for the efficient secretion of procollagen (Graham et al. 1995). Ascorbic acid is a crucial cofactor for the enzymes prolyl hydroxylase and lysyl hydroxylase, both major posttranslational modifiers of procollagen in the endoplasmic reticulum. While hydroxylation

of the frequent lysyl residues in collagen plays a role later for collagen crosslinking, hydroxylation of the prolyl residues in procollagen immediately determines its thermal stability and thus its folding state (Yamauchi and Sricholpech 2012). The term procollagen refers to a triple helix composed of three procollagen alpha chains. In the endoplasmic reticulum, three such procollagen alpha chains form a procollagen triple helix. The assembly of the three alpha chains in a triple helix starts at the C-terminus, in a zipper-like fashion, toward the N-terminus. This process takes roughly a quarter of an hour for one triple helix (Raghunath et al. 1994). During folding, several enzymes posttranslationally modify the nascent triple helix. Because a folded triple helix is not easily accessible to these enzymes, they are "surfing" along the procollagen alpha chains ahead of the folding front to fulfill their tasks. All biochemical processes are in equilibrium. This means for collagen triple helices that they are continuously folding and unfolding ("breathing"). As only a completely folded procollagen can leave the endoplasmic reticulum, the equilibrium at body temperature must be shifted to the fully folded state. Here, prolyl hydroxylation comes into play by generating hydroxyprolines that help locking the triple helix in the folded state. In the absence of ascorbic acid, collagen biosynthesis can still occur but under-hydroxylated procollagen populations will be retained within the cell because they are not properly folded. It follows that ascorbic acid is crucial for an efficient collagen secretion (Barnes 1975). Vitamin C cannot be synthesized by humans, making it an essential vitamin that must be ingested. Nutritional Vitamin C deficiency, known as scurvy, leads to a paucity of extracellular collagen. This has dramatic effects on wound healing, blood vessel integrity, and periodontal health, all pointing to severe impairment of ECM maintenance (Peterkofsky 1991). It was estimated that British troops "even in World War I had more scurvy than gunshot casualties" (Kiple 2002) and that prior to the twentieth century, more soldiers and sailors deployed in warfare succumbed to scurvy than died in battle or accidents (McDowell 2013).

While scurvy might now be less frequent in modern societies, it is often involuntarily reproduced in ascorbic acid-free cell cultures where we find cells intracellularly retaining their procollagen, while the little that is secreted extracellularly hardly forms a pericellular matrix. The sensitivity to under-hydroxylation appears to vary between collagen and cell types and may depend on the percentage of hydroxylated prolyl residues per procollagen alpha chain and the specific thermostability pattern of the collagen type in question. For collagen I, a minimum of 120 hydroxylated prolyl residues is required to achieve thermostability (Royce and Steinmann 2003). While collagen I and VII may be the most sensitive to vitamin C deficiency in vitro, observations with collagen IV deposition suggest that it might be less vulnerable, but no systematic studies exist here. While MMC facilitates ECM deposition, it also depends on full collagen secretion to unlock its greatest benefits in building a "homemade" ECM microenvironment for cells in culture. Other ECM components are not dependent on the role of ascorbic acid such as fibronectin or elastic microfibrillar components. Therefore, an ECM built in the absence of vitamin C would preferably contain non-collagenous components and therefore present with a skewed stoichiometry. This, however, also would offer an opportunity of modulating ECM compositions using MMC.

5 Macromolecular Crowding in Macromolecular Assemblies

Collagen gels can be generated in vitro exploiting the intrinsic self-assembly process of collagen triple helices. Solutions of collagen containing pretrimmed procollagen can be made or bought. Collagen molecules are prevented from fibril formation by keeping them at very low pH, for example, in 1 M hydrochloric acid or 0.5 M acetic acid. When this solution is brought to neutral pH, the collagen assembly process begins rapidly, resulting in the formation of a gel made of collagen fibrils in a haphazard arrangement. This way of making collagen hydrogels is very popular, and collagen gels are used for a variety of assays and culture systems (Collin et al. 2011). The process of gel formation, which is essentially a process of fibrillogenesis, can be followed via turbidimetry. Light is shone through a cuvette containing the collagen solution; when fibrillogenesis occurs, the light is scattered because of the forming fibrils that render the solution turbid. Collagen gel formation is described in turbidimetry by two phases. Firstly, multiple foci form, consisting of a few collagen triple helices forming nuclei. This is called the lag phase, and while there is no apparent signal in turbidimetry, the formation of numerous nuclei is happening. Then, the solution starts getting turbid rapidly because of the nuclei that are now elongated into linear structures forming proper fibrils. This is the growth phase. The absorption curve shows a steep slope during this phase and a plateau when all collagen triple helices are spent in the process of fibrillogenesis (Dewavrin et al. 2015) (Fig. 2). When we first experimented with negatively charged macromolecular crowders (e.g., dextran sulfate), gel formation would occur so rapidly that turbidimetry was impossible to do (unpublished). We then studied the effects of single neutrally charged crowders to capture changes of fibril formation quantitatively (Dewavrin et al. 2015, 2014). MMC appeared to shorten the lag phase of collagen fibrillogenesis by creating a greater number of elongation nuclei in a shorter time window while the rate and extent of elongation (visible as the slope) remained unchanged. However, a plateau was reached faster, which suggested that crowding leads to the formation of more but thinner collagen fibrils (Fig. 2). These cell-free in vitro data on collagen fibrillogenesis were later confirmed in monolayer cell culture systems with the basement membrane collagen IV in the adipogenic differentiation of human bone marrow-derived stromal cells and progenitors from the stromal vascular fraction of human subcutaneous fat. Here, brown fat cells generated under macromolecular crowding were embedded in a collagen IV cocoon consisting of thin fibrils, whereas non-crowded cultures showed coarse and thick collagen bundles framing but not enveloping cells (Lee et al. 2016) (Fig. 3).

The assembly of collagen has now been extensively studied under macromolecular crowding in cell-free systems and in monolayer cultures of collagen-producing cells. The primordial ECM molecule, however, is fibronectin. It is produced and laid down first by cells (and or grabbed from serum as discussed above) and serves as a template for collagen deposition (Kadler et al. 1996). While we have not systematically studied fibronectin ECM formation in vitro, we have shown that macromolecular crowding does promote fibronectin deposition in vitro in mesenchymal stem cells seeded in the absence of serum (Chen et al. 2011). However, more work needs to be done to study self-assembly of fibronectin under MMC.

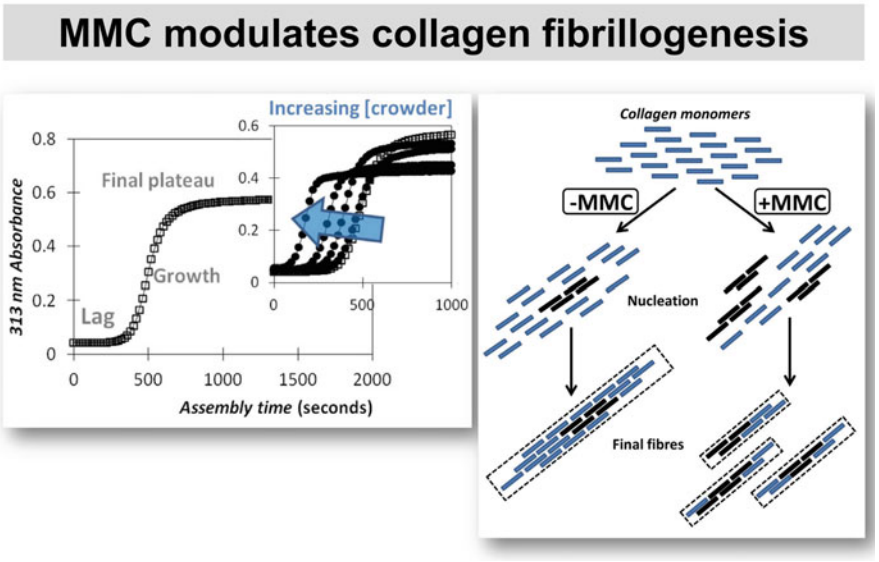

Fig. 2 Effects of mono-crowding using 400 kDa on the kinetics of collagen I gel formation. The starting material is an acidic solution containing single collagen triple helices. Upon neutralization of this solution, the collagen fiber assembly starts and a collagen gel forms. Left panel: phases of collagen assembly as assessed by turbidimetry at 313 nm: No apparent signal is obtained before and during nucleation (lag time). The phase of elongation of nuclei (fiber growth) becomes visible with an increased absorption and a final A313 plateau, denoting the conclusion of collagen assembly. Right panel: the generation of an increased number of nuclei under MMC leads to the formation of more but finer collagen fibers. (Reprinted from Dewavrin et al. 2014, with permission from Elsevier)

We have shown that MMC promotes ECM formation at least on two levels: on the enzymatic level and on the supramolecular assembly level (see Figs. 1 and 2). Thus, the logic of applying macromolecular crowding to empower cells to build their own microenvironment appears quite compelling. While adding macromolecular crowders to culture medium is straightforward, the relevant biophysics, however, are highly complex and have been subject to in vitro and extensive in silico considerations. These considerations can be found in more detail in a number of pertinent publications, some of which are cited throughout this text. One of these considerations is that in any given volume, macromolecular crowders occupy space. If we assume that all molecules in such a given volume are spherical and that we know their number (molarity) and their size (hydrodynamic radius, R_H), we can then calculate the volume they occupy and set this in relation to the given volume they are in. Thus, the fractional volume occupancy (FVO) with the mathematical symbol Ψ can be determined, allowing a first approximation of crowdedness in a given system. Biological systems show a Ψ in the range of 5–32% and are expressed as volume/volume (Fig. 4) (Chen et al. 2011).

For all intents and purposes, we shall equate this FVO with the excluded volume, which means that the crowder-occupied space excludes all other molecules from that space. This, of course, only applies for truly globular molecules and is a generalization

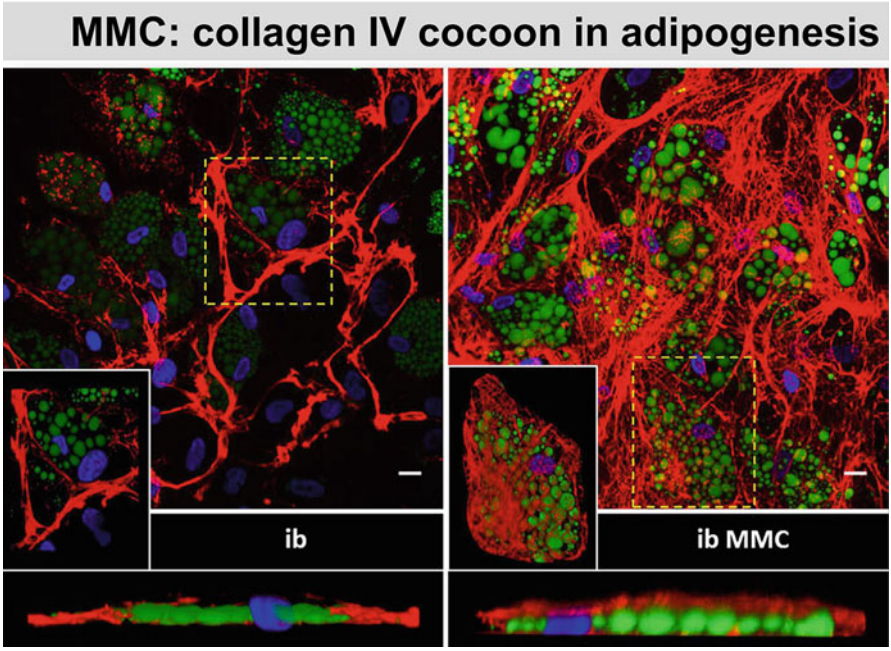

Fig. 3 MMC enhances formation of Col IV basement membrane architecture during adipogenic differentiation. Human bone marrow-derived stem cells were differentiated into brown adipocytes under standard culture conditions (left panels) and under mixed macromolecular crowding using Ficoll (right panels). Z-stack images were obtained for each condition through confocal microscopy. The Z-project images show nuclei (blue), Col IV (red), and lipid droplets (green). The inserts and cross-sections are the reconstructed 3D images of the selected cells boxed in yellow to show the pericellular distribution of Col IV. Scale bar: 20 μm. Bottom images represent cross-sections of the region of interest. The cross-section reveals that only under MMC single adipocytes are fully wrapped in a collagen IV cocoon. This situation would be normally seen only in cells seeded into hydrogels. Thus, MMC creates an ultra-flat 3D setting with regard to ECM distribution in a monolayer. (Reprinted from Lee et al. 2016, under Creative Commons CC-BY-4.0 license. Available from: https://doi.org/10.1038/srep21173)

that will not characterize all macromolecules well. For example, a collagen triple helix is a rod-like trimer with a diameter of 1.5 nm and a length of 280 nm, as aspherical as a molecule can get (Shoulders and Raines 2009; Lodish et al. 2000). However, assumptions must be made to get to grips with any system, and this approach has a time-honored tradition in engineering. Basically, the power of macromolecular crowding resides in its ability to confine reactants to less space, thereby leading to a virtual increase in concentration of reactants. However, macromolecular crowding does much more than just making reaction volumes smaller or reducing the volume of culture media. We have seen a moderate increase of collagen deposition in greatly reduced culture medium (200 μl versus 500 μl) in 24-well plates (Chen et al. 2011). While we created a concentration effect in these cell cultures, we put them in danger of drying out or running out of nutrients fast. Moreover, just reducing the culture medium volume

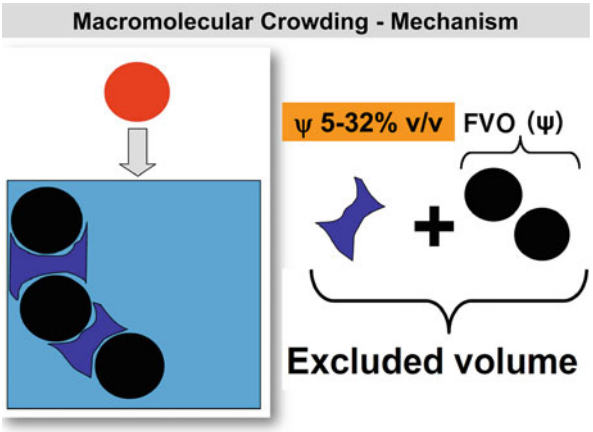

Fig. 4 A simplified representation of the generation of the excluded volume effects through the presence of macromolecular crowders in culture medium. Here, a test molecule (red) is about to enter a given volume (blue box). The given volume already contains three macromolecules (crowders, black). If we assume that these crowders occupy 30% of this volume, their fractional volume occupancy (*FVO*, Ψ) would be given as 30% (v/v). The FVO can be calculated when the volume is known, along with the number of crowders (molarity) and their volume. The volume can be calculated assuming the crowders to be spherical. In this case, the hydrodynamic radius will be used. The additional unavailable volume is a challenge to calculate, as several factors such as electrostatic repulsion and hydration shell need to be considered. Water molecules and Brownian motion are not considered in this model. (Reprinted from Chen et al. 2011, with permission from Elsevier)

will not change the relationship between total volume and available volume. In contrast, with a crowded standard culture medium volume (500 µl), we saw much stronger deposition effects because the thermodynamic activity is much larger in a crowded culture medium, which has simply to do with the substantially reduced available volume versus a total given volume (Fig. 5).

6 The Power of Charge and More: Mixed Macromolecular Crowding

Macromolecular crowding can be created using a single size species macromolecule, and most model systems work with one crowder; however, the combination of two or more crowders is more powerful than a system with only one crowder. A selection of commonly used crowders can be found in Table 1. We shall use the term "mono-crowding" for usage of only one size of one particular macromolecular crowder and "mixed macromolecular crowding" for usage of two or more crowders with different molecular weights. The molecular weight, Mw, of a given crowding molecule would cause steric hindrance by occupying space. At physiological pH, most proteins are negatively charged, depending on their amino acid composition and their respective

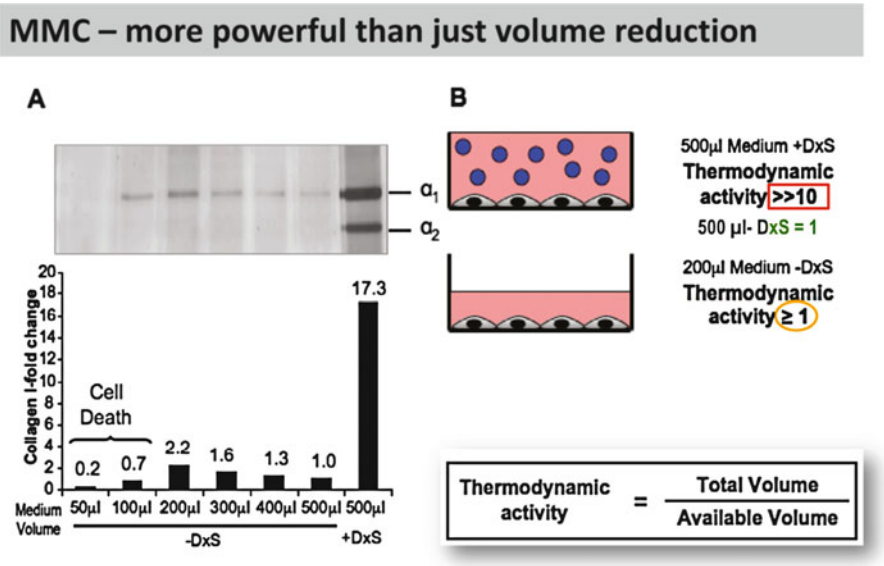

Fig. 5 Medium volume reduction effects are negligible compared with macromolecular crowding of the culture medium. (**a**) Fibroblast cultures were grown under non-crowded conditions in decreasing medium volumes. Their collagen matrix deposition was compared with cells in the highest volume under MMC with dextran sulfate (*DxS*). A 2.2-fold increase of collagen deposition was indeed seen in 200 µl but was clearly outperformed in DxS-crowded fibroblasts (17-fold increase) in 500 µl. In 100 µl and 50 µl, respectively, wells dried out, resulting in cell death. (**b**) The ratio between total volume and available volume will always stay 1 under non-crowded conditions, regardless of the actual volume in the cell culture as both volumina do not change in their relationship to each other. For 500 µl standard culture, the ratio is 1 (green text), as well as in 200 µl. However, it could be argued that in a smaller volume, cellular secretion of macromolecules might introduce some form of MMC, as well, so reducing the available volume slightly. However, thermodynamic activities in 500 µl of DxS-crowded medium increase dramatically, as the available volume is substantially reduced under MMC. (Copyright 2010 Peng Y, Raghunath M. Published in (Peng and Raghunath 2010) and reproduced with permission under CC-BY-NC-SA 3.0 license. Available from: https://doi.org/10.5772/8573)

cumulative isoelectric points. Consequently, a negatively charged crowder would have an even greater volume-excluding effect due to electrostatic repulsion of negatively charged molecules by negatively charged crowders (Harve et al. 2006). In fact, we have demonstrated that the hydrodynamic radius (R_H) of negatively charged crowders is substantially larger in physiological salt solutions than a comparably sized neutrally charged crowder (Lareu et al. 2007b). The resulting larger volume-excluding effect that virtually increases reactant concentration could explain the extremely accelerated collagen deposition seen in presence of dextran sulfate 500 kDa (Lareu et al. 2007a, b). The size differences, as described by the hydrodynamic radius, also explained why we saw much weaker effects with mono-crowding with neutrally charged dextran or Ficoll molecules. Full collagen deposition under dextran sulfate was effectively enhanced in vitro within 48 h with fetal lung

Table 1 Commonly used macromolecular crowders for mono- and mixed crowding, cell types analyzed, and overview of relevant outcomes

Crowder	Cell types tested	Outcome	Reference
Dextran sulfate 10 kDa	WI-38 fibroblasts	Accelerated conversion of procollagen to collagen	Lareu et al. (2007a)
Dextran sulfate 500 kDa	WI-38, WS-1, corneal, dermal, and hypertrophic scar fibroblasts	Increased ECM deposition, accelerated C-propeptide cleavage, myofibroblastic differentiation of corneal fibroblasts	Bateman and Golub (1990), Lareu et al. (2007a, b), Chen et al. (2009), Satyam et al. (2014), and Kumar et al. (2015b)
Dextran T40	Dermal fibroblasts	Full processing of procollagen to collagen	Bateman et al. (1986) and Jukkola et al. (1991)
Dextran 670 kDa	WI-38 and hypertrophic scar fibroblasts	Increased ECM deposition	Lareu et al. (2007b)
Polystyrene sulfonate 200 kDa	WI-38 fibroblasts	Increased ECM deposition	Lareu et al. (2007a)
Polyethylene glycol 4 kDa	Dermal fibroblasts	Full processing of procollagen to collagen	Bateman et al. (1986)
Polyvinylpyrrolidone 40 kDa, 360 kDa	Bone marrow stem cells, dermal fibroblasts	Increased ECM deposition, increased cell proliferation (with 360 kDa)	Rashid et al. (2014)
Carrageenan	WI-18 and WS-1 fibroblasts, bone marrow stem cells	Increased ECM deposition modulated by crowder polydispersity	Satyam et al. (2016, 2014), and Cigognini et al. (2016)
Ficoll 70 kDa	WI-38 fibroblasts	No increase in ECM deposition	Lareu et al. (2007a)
Ficoll 400 kDa	WI-38 fibroblasts	No increase in ECM deposition. Modulation of fibrillogenesis kinetics of reconstituted kidney ECM	Lareu et al. (2007a) and Magno et al. (2017)
Cocktail of Ficoll 70 kDa and 400 kDa	Bone marrow stem cells, chondrocytes, WI-38, WS-1, and corneal fibroblasts	Increased collagen deposition, increased glycosaminoglycan deposition	Chen et al. (2009), Satyam et al. (2014), Rashid et al. (2014), Cigognini et al. (2016), Zeiger et al. (2012), Ang et al. (2013), and Kumar et al. (2015a)
Cocktail of dextran sulfate 10 kDa, Ficoll™ 70 kDa and Ficoll™ 400 kDa	Bone marrow stem cells	Increased deposition of ECM and growth factor retention	Prewitz et al. (2015)

fibroblast lines, as more collagen was detected within the matrix, as compared to cells cultured for several weeks without macromolecular crowding (Lareu et al. 2007b; Chen et al. 2009).

However, the deposited collagen (and fibronectin) matrix appeared granular; ultrastructural imaging revealed these granules to contain thin cross-striated collagen fibrils, but a contiguous collagen meshwork was not apparent under negative mono-crowding, (Lareu et al. 2007b). We also came to realize that not every cell line thrives under these crowding conditions. We suspected that dextran sulfate, and likewise other negatively charged crowders, might interfere with a variety of growth factors that have a positive net charge. We initially saw weaker results using single neutrally charged crowders (Lareu et al. 2007a) but were inspired by modeling work predicting that the combination of two differently sized crowders would generate a stronger volume occupancy/exclusion effect (Zhou 2008). We therefore combined the sucrose copolymers Ficoll 70 kDa with Ficoll 400 kDa and after a few titration rounds determined that mixed macromolecular crowding had indeed a strong, albeit slower than dextran sulfate, effect on collagen deposition (Lareu et al. 2007a; Chen et al. 2009). The current Ficoll mixture we have been using ever since has a theoretical FVO of 18% v/v, which we determined to be equivalent to the albumin fraction in serum (Chen et al. 2011b).

Using cell-free collagen assembly as a readout system, we could experimentally confirm previous predictions (Zhou 2008) that the combination of a minimum of two differently sized species of crowders indeed creates more excluded volume than theoretically expected and discussed the underlying theory (Fig. 6) (Dewavrin et al. 2015). We have used mixed macromolecular crowding successfully also in settings of enzymatic reactions, such as PCR (Lareu et al. 2007a) and in DNA hybridization experiments (Harve et al. 2010). The advantage of mixed macromolecular crowding using Ficoll is that two synthetic polymers of defined size give a controlled biphasic size distribution pattern. However, theoretical and experimental data would suggest that a third or fourth crowder in descending size might even close more spaces. Indeed we used triple crowding in PCR and hybridization experiments (Harve et al. 2010). Therefore, reproducible polydispersity would be an interesting approach, as it would represent a bandwidth of different sizes. This approach has been put into practice using carrageenan, a polydisperse macromolecule that has shown powerful ECM deposition in vitro (Satyam et al. 2014; Kumar et al. 2015a, b; Cigognini et al. 2016; Satyam et al. 2016; Kumar et al. 2018). Therefore, mixed macromolecular crowding should be attempted in vitro wherever possible (Fig. 6).

7 Cell Sheet Engineering and Macromolecular Crowding: A New Match

Cell sheet engineering is based on growing cell layers on a thermosensitive polymer at body temperature and then to lift the cells off, along with their deposited ECM, as one coherent layer when the culture vessel is placed in a

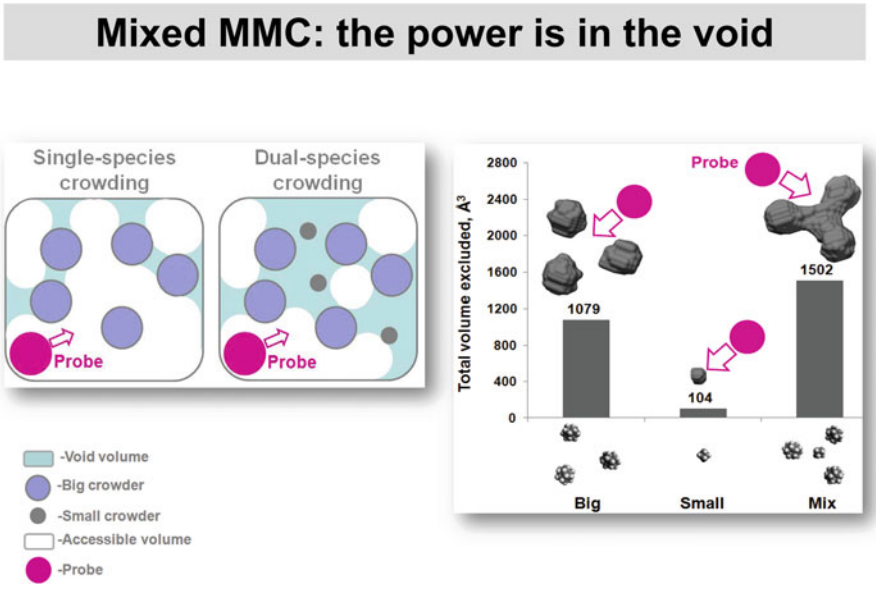

Fig. 6 Modelling of mixed macromolecular crowding. Left: Comparison to the volume that is accessible to a probe in a volume that is occupied by one species of crowders (single) or two different species (dual). In the mixed condition, the small crowder does not occupy a significant volume but reduces the average distance between larger crowders to a value lower than the probe diameter: a void volume is created. The total excluded volume is therefore greater than the sum of the crowders' volumes, as the small crowder is granted with extra volume occupancy (the void volume) due to its proximity to the big crowders. Right: molecular modeling reveals that the total void volume generated by mixed MMC is larger than the sum of void volumes generated by either crowding component. Thus, mixing crowders is a powerful approach to tune an aqueous system. (Reprinted with permission from Dewavrin et al. 2015. Copyright (2015) American Chemical Society)

cooler environment (Yang et al. 2005; Yamato and Okano 2004). This feat is achieved by coating tissue culture polystyrene with poly (N-isopropylacrylamide) (variously abbreviated PNIPAAm). At a certain temperature, such as 37 °C, PNIPAAm strands are coiled up, but at lower temperatures, like 20 °C, the coils unwind. Cell seeded onto PNIPAAm would find a smooth adhesive surface at 37 °C and then would find themselves pushed away from underneath as the polymer uncoils when the culture medium cools down outside of the incubator (Yamato et al. 2007). The chemistry behind this material is based on its glass transition temperature (Rollason et al. 1993; von Recum et al. 1998). In conditions of low ECM deposition, these thermosensitive polymers work well. However, under macromolecular crowding conditions, where abundant ECM is deposited, copolymers of PNIPAAm are necessary to detach intact and ECM-rich tissue equivalents (Satyam et al. 2014).

8 Impact of Macromolecular Crowding on Three-Dimensional Extracellular Matrix Networks and Associated Cell Signaling Events

2D and 3D in vitro systems are extensively used in tissue engineering and regenerative medicine (Shologu et al. 2016; Li and Kilian 2015). Macromolecular crowding contributes to a blurring of the boundaries between what is called "2D" and "3D" cell culture. Traditionally, the term 3D tissue engineering is used to describe thick, tissue-like constructs, often cells seeded onto a scaffold. We posit that there is a clear Z-dimension (height) in rich in ECM, scaffold-free, crowded cultures, which better imitates the in vivo-like cell microenvironment without the shortcomings that typical 3D systems present in terms of microscopic analysis and viability assessment.

Confocal imaging analysis suggested that adipocytes generated from mesenchymal stem cells under MMC were covered and embedded in ECM, resembling a layered system with cells embedded between layers of ECM (Lee et al. 2016; Bolevich et al. 2016) (Fig. 7). With more collagen available around them, due to macromolecular crowding, the cells can generate more interfaces with the ECM and especially more attachment points, even on the top of the cells. This was graphically demonstrated by the presence of focal adhesions signifying an increased cell-matrix engagement under crowding conditions (Lee et al. 2016). With more focal adhesions engaging a richer ECM, the integrin-mediated signaling is likely to increase and even change, as cells are integrative systems which constantly process and combine various extracellular signals to adapt their behavior in response to external stimuli. This three-dimensional architecture of the ECM technology has also enabled investigations into the intrinsic brown potential of adipogenic differentiation of bone marrow-derived stem cells and progenitors from the stromal vascular fraction of subcutaneous fat (Lee et al. 2016). A similar 3D ECM network surrounding adipocytes has been observed in vivo by electron microscopy of native adipose tissue (Flynn 2010; Sbarbati et al. 1987). In vitro, this situation is usually found only in cells embedded in artificially created 3D matrices (Polacheck et al. 2014). This means that a monolayer culture system can be turned around into an ultra-flat 3D system via macromolecular crowding, a consideration that is important for the design of cell-based assays for the development of drug screening tests.

As we have seen, MMC can induce a variety of cells to deposit much more ECM than under standard conditions and with higher complexity than artificial/engineered matrices. This offers the opportunity to develop these rich in ECM cell constructs as an optimal niche for in vitro expansion of various cell types, following decellularization. For example, human embryonic stem cells benefit from a human fibroblast matrix deposited under crowding conditions and can be passaged effectively on such cell-produced matrix (Peng et al. 2012). The most recent example of the successful application of MMC was the generation of (decellularized) bone marrow matrix for the cultivation of hematopoietic stem cells, applying mixed MMC with novel crowder combinations (Prewitz et al. 2015). The application of crowding to produce the full depositional cascade of collagen in complete living

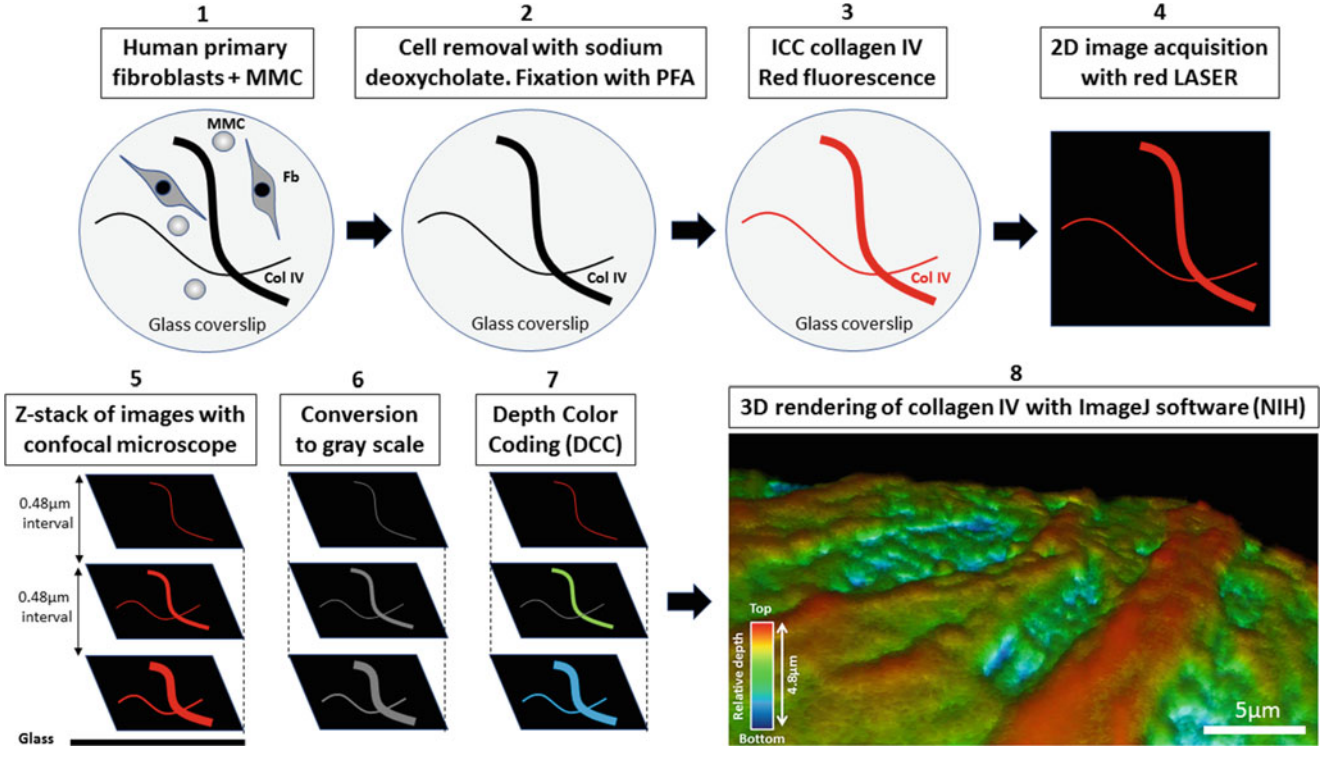

Fig. 7 3D rendering of an ECM of collagen IV produced by human primary fibroblasts upon macromolecular crowding (*MMC*). (1) Human primary fibroblasts (*Fb*) were seeded on glass coverslips and incubated in the presence of crowders (MMC) resulting in an enhanced deposition of collagen IV (*Col IV*). (2) The cells were removed with sodium deoxycholate. The samples were then washed and fixed with PFA. (3) Collagen IV was stained by immunocytochemistry (*ICC*). (4) The samples were then imaged in 2D using a red LASER. (5) The samples were then imaged plane by plane (Z-stack) using an inverted confocal microscope

fibroblast monolayers has led to the construction of a high content screening tool, the scar in a jar, for antifibrotic compound screening (Chen et al. 2009; Chen and Raghunath 2009).

9 Conclusion

Cells in tissue culture media basically represent seawater with some additional ingredients including a fraction of fetal calf serum. This makes for a very dilute environment when compared with the crowded intra- and extracellular milieus. This ocean of dilution leads to a number of biochemical and biophysical processes to occur with less speed and efficiency than they would in vivo. It is often claimed that in vitro cell culture is artificial to start with and therefore is of limited physiological relevance. While this argument is often used to make a case for animal experimentation, it should not be an excuse for not improving cell culture conditions. We believe that much can be done to move in vitro cell culture systems closer to human physiology by correcting some deficiencies, such as too much oxygen, lack of appropriate surface signals, the absence of biomechanical stimulation and macromolecular crowding, and a deficient microenvironment. It is worthwhile considering that the need for 3D tissue-engineered systems, living organ equivalents, is increasing in industry. There is societal pressure to abandon animal experimentation which is increasingly translating into political pressure and, therefore, legislation to enforce the 3R of animal welfare. Within this framework, tissue-engineered tissue equivalents will see a continued demand as long as they are robust and can be validated. In this context, macromolecular crowding can be a very valuable tool for not only answering fundamental questions but also increasing the functionality of cells in vitro by facilitating faster and deeper differentiation of progenitor cells or greater phenotypic stabilization, all via the generation of a complex human ECM.

Acknowledgments This work was supported from the Health Research Board; Health Research Awards Programme, under the grant agreement number HRA_POR/2011/84; and the Science Foundation Ireland, under the grant agreement number 15/CDA/3629. This publication has also been supported from the Science Foundation Ireland and the European Regional Development Fund, under the grant agreement number 13/RC/2073. The authors would also like to acknowledge the Tendon Therapy Train, H2020-MSCA-ITN-2015 Programme, grant agreement number 676338. M.R. acknowledges the support of start-up funding from the Zurich University of Applied Sciences

Fig. 7 (continued) (Zeiss LSM510) with an interval of 0.48 μm between two successive optical planes. (6) All images were converted into gray scale preserving pixel intensity but removing the original colors. (7) Each image was given an artificial color according to its position in the Z (vertical) axis (Depth Color Coding, *DCC*). Images acquired at the bottom of the sample (near the glass coverslip) were colored in blue. Images acquired on the top of the sample were artificially colored in red. (8) The stack of color-coded pictures was then imported into ImageJ software (NIH) for 3D rendering. (For more information on the Material and Methods, refer to Benny et al. 2015, PMID: 25058150)

(9710.3.13.5.0146.01) and Biotechnet (NTN Swiss Biotech) support via the competence Center TEDD (Tissue Engineering for Drug Development and Substance Testing) and acknowledges membership in the EU COST Action Programme CellFit CA16119.

References

Ahmed N (2005) Advanced glycation endproducts – role in pathology of diabetic complications. Diabetes Res Clin Pract 67(1):3–21

Alford AI, Hankenson KD (2006) Matricellular proteins: extracellular modulators of bone development, remodeling, and regeneration. Bone 38(6):749–757

Ang XM, Lee MH, Blocki A, Chen C, Ong LS, Asada HH, Sheppard A, Raghunath M (2013) Macromolecular crowding amplifies adipogenesis of human bone marrow-derived mesenchymal stem cells by enhancing the pro-adipogenic microenvironment. Tissue Eng A 20:966–981

Ang XM, Lee MH, Blocki A, Chen C, Ong LL, Asada HH, Sheppard A, Raghunath M (2014) Macromolecular crowding amplifies adipogenesis of human bone marrow-derived mesenchymal stem cells by enhancing the pro-adipogenic microenvironment. Tissue Eng A 20(5–6):966–981

Aplin A, Howe A, Alahari S, Juliano R (1998) Signal transduction and signal modulation by cell adhesion receptors: the role of integrins, cadherins, immunoglobulin-cell adhesion molecules, and selectins. Pharmacol Rev 50(2):197–264

Arigony ALV, de Oliveira IM, Machado M, Bordin DL, Bergter L, Prá D, Pêgas Henriques JA (2013) The influence of micronutrients in cell culture: a reflection on viability and genomic stability. Biomed Res Int 2013:597282

Barnes M (1975) Function of ascorbic acid in collagen metabolism. Ann N Y Acad Sci 258(1):264–277

Barnes D, Sato G (1980) Serum-free cell culture: a unifying approach. Cell 22(3):649–655

Bateman JF, Golub SB (1990) Assessment of procollagen processing defects by fibroblasts cultured in the presence of dextran sulphate. Biochem J 267:573–577

Bateman JF, Cole WG, Pillow JJ, Ramshaw J (1986) Induction of procollagen processing in fibroblast cultures by neutral polymers. J Biol Chem 261(9):4198–4203

Benecke B-J, Ben-Ze'ev A, Penman S (1978) The control of mRNA production, translation and turnover in suspended and reattached anchorage-dependent fibroblasts. Cell 14(4):931–939

Benny P, Badowski C, Lane EB, Raghunath M (2015) Making More Matrix: Enhancing the deposition of dermal-epidermal junction components in vitro and accelerating organotypic skin culture development, using macromolecular crowding, Tissue Engineering A, 21 (1–2):183–192

Bettger WJ, McKeehan WL (1986) Mechanisms of cellular nutrition. Physiol Rev 66(1):1–35

Birk DE, Fitch J, Babiarz J, Doane K, Linsenmayer T (1990) Collagen fibrillogenesis in vitro: interaction of types I and V collagen regulates fibril diameter. J Cell Sci 95(4):649–657

Birnie J, Grayson J (1952) Observations on temperature distribution and liver blood flow in the rat. J Physiol 116(2):189

Bolevich S, Kogan AH, Zivkovic V, Djuric D, Novikov AA, Vorobyev SI, Jakovljevic V (2016) Protective role of carbon dioxide (CO_2) in generation of reactive oxygen species. Mol Cell Biochem 411(1–2):317–330

Bonnans C, Chou J, Werb Z (2014) Remodelling the extracellular matrix in development and disease. Nat Rev Mol Cell Biol 15(12):786–801

Bornstein P (1974) The structure and assembly of procollagen – a review. J Supramol Struct 2(2–4):108–120

Brownlee M (2001) Biochemistry and molecular cell biology of diabetic complications. Nature 414(6865):813–820

Cetta G, Rossi A, Tenni R, Valli M, Forlino A, Zanaboni G, Dyne K, Burgio GR (1993) Deposition of mutant type I collagen in the extracellular matrix of cultured dermal fibroblasts in osteogenesis imperfecta. Connect Tissue Res 29(1):41–49

Chen CZ, Raghunath M (2009) Focus on collagen: in vitro systems to study fibrogenesis and antifibrosis state of the art. Fibrogenesis Tissue Repair 2:7

Chen SS, Fitzgerald W, Zimmerberg J, Kleinman HK, Margolis L (2007) Cell-cell and cell-extracellular matrix interactions regulate embryonic stem cell differentiation. Stem Cells 25(3):553–561

Chen CZ, Peng YX, Wang ZB, Fish PV, Kaar JL, Koepsel RR, Russell AJ, Lareu RR, Raghunath M (2009) The scar-in-a-jar: studying potential antifibrotic compounds from the epigenetic to extracellular level in a single well. Br J Pharmacol 158(5):1196–1209

Chen C, Loe F, Blocki A, Peng Y, Raghunath M (2011) Applying macromolecular crowding to enhance extracellular matrix deposition and its remodeling in vitro for tissue engineering and cell-based therapies. Adv Drug Deliv Rev 63(4):277–290

Cigognini D, Lomas A, Kumar P, Satyam A, English A, Azeem A, Pandit A, Zeugolis D (2013) Engineering in vitro microenvironments for cell based therapies and drug discovery. Drug Discov Today 18(21–22):1099–1108

Cigognini D, Gaspar D, Kumar P, Satyam A, Alagesan S, Sanz-Nogués C, Griffin M, O'Brien T, Pandit A, Zeugolis DI (2016) Macromolecular crowding meets oxygen tension in human mesenchymal stem cell culture – a step closer to physiologically relevant in vitro organogenesis. Sci Rep 6:30746. https://doi.org/10.1038/srep30746

Collin EC, Grad S, Zeugolis DI, Vinatier CS, Clouet JR, Guicheux JJ, Weiss P, Alini M, Pandit AS (2011) An injectable vehicle for nucleus pulposus cell-based therapy. Biomaterials 32(11):2862–2870

Curtis A, Forrester J, McInnes C, Lawrie F (1983) Adhesion of cells to polystyrene surfaces. J Cell Biol 97(5):1500–1506

Damsky C (1999) Extracellular matrix–integrin interactions in osteoblast function and tissue remodeling. Bone 25(1):95–96

Dewavrin JY, Hamzavi N, Shim VP, Raghunath M (2014) Tuning the architecture of three-dimensional collagen hydrogels by physiological macromolecular crowding. Acta Biomater 10(10):4351–4359

Dewavrin JY, Abdurrahiem M, Blocki A, Musib M, Piazza F, Raghunath M (2015) Synergistic rate boosting of collagen fibrillogenesis in heterogeneous mixtures of crowding agents. J Phys Chem B 119(12):4350–4358

Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D, Deans R, Keating A, Prockop D, Horwitz E (2006) Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy 8(4):315–317

Eagle H (1955) Nutrition needs of mammalian cells in tissue culture. Science 122:501–504

Ellis RJ (2001) Macromolecular crowding: obvious but underappreciated. Trends Biochem Sci 26(10):597–604

Fleischmajer R, Perlish J, Burgeson R, Shaikh-Bahai F, Timpl R (1990) Type I and type III collagen interactions during fibrillogenesis. Ann N Y Acad Sci 580(1):161–175

Flynn LE (2010) The use of decellularized adipose tissue to provide an inductive microenvironment for the adipogenic differentiation of human adipose-derived stem cells. Biomaterials 31(17):4715–4724

Gelse K, Pöschl E, Aigner T (2003) Collagens – structure, function and biosynthesis. Adv Drug Deliv Rev 55(12):1531–1546

Graham MF, Willey A, Adams J, Yager D, Diegelmann RF (1995) Role of ascorbic acid in procollagen expression and secretion by human intestinal smooth muscle cells. J Cell Physiol 162(2):225–233

Gregoire FM, Smas CM, Sul HS (1998) Understanding adipocyte differentiation. Physiol Rev 78(3):783–809

Grinnell F, Feld MK (1979) Initial adhesion of human fibroblasts in serum-free medium: possible role of secreted fibronectin. Cell 17(1):117–129

Gstraunthaler G, Lindl T, van der Valk J (2013) A plea to reduce or replace fetal bovine serum in cell culture media. Cytotechnology 65(5):791–793

Harve KS, Raghunath M, Lareu RR, Rajagopalan R (2006) Macromolecular crowding in biological systems: dynamic light scattering (DLS) to quantify the excluded volume effect (EVE). Biophys Rev Lett 1(3):317–325

Harve KS, Lareu R, Rajagopalan R, Raghunath M (2010) Understanding how the crowded interior of cells stabilizes DNA/DNA and DNA/RNA hybrids–in silico predictions and in vitro evidence. Nucleic Acids Res 38(1):172–181

Hayman EG, Pierschbacher MD, Suzuki S, Ruoslahti E (1985) Vitronectin – a major cell attachment-promoting protein in fetal bovine serum. Exp Cell Res 160(2):245–258

Hoffman W, Charbel F, Gonzalez Portillo G, Edelman G, Ausman J (1998) Regional tissue pO_2 pCO_2, pH and temperature measurement. Neurol Res 20(sup1):S81–S84

Hojima Y, Behta B, Romanic AM, Prockop DJ (1994) Cleavage of type I procollagen by *C*- and *N*-proteinases is more rapid if the substrate is aggregated with dextran sulfate or polyethylene glycol. Anal Biochem 223(2):173–180

Honn KV, Singley JA, Chavin W (1975) Fetal bovine serum: a multivariate standard. Proc Soc Exp Biol Med 149(2):344–347

Jukkola A, Risteli J, Risteli L (1991) Effect of dextran on synthesis, secretion and deposition of type III procollagen in cultured human fibroblasts. Biochem J 279:49–54

Kadler KE, Holmes DF, Trotter JA, Chapman JA (1996) Collagen fibril formation. Biochem J 316(Pt 1):1–11

Kielty CM, Grant ME (2003) The collagen family: structure, assembly, and organization in the extracellular matrix. In: Connective tissue and its heritable disorders. Wiley, Hoboken, pp 159–221

Kiple KF (2002) The Caribbean slave: a biological history. Cambridge University Press, Cambridge

Kumar P, Satyam A, Fan X, Collin E, Rochev Y, Rodriguez BJ, Gorelov A, Dillon S, Joshi L, Raghunath M, Pandit A, Zeugolis DI (2015a) Macromolecularly crowded in vitro microenvironments accelerate the production of extracellular matrix-rich supramolecular assemblies. Sci Rep 5:8729. https://doi.org/10.1038/srep08729

Kumar P, Satyam A, Fan X, Rochev Y, Rodriguez BJ, Gorelov A, Joshi L, Raghunath M, Pandit A, Zeugolis DI (2015b) Accelerated development of supramolecular corneal stromal-like assemblies from corneal fibroblasts in the presence of macromolecular crowders. Tissue Eng Part C 21(7):660–670

Kumar P, Satyam A, Cigognini D, Pandit A, Zeugolis DI (2018) Low oxygen tension and macromolecular crowding accelerate extracellular matrix deposition in human corneal fibroblast culture. J Tissue Eng Regen Med 12:6–18

Kyburz KA, Anseth KS (2015) Synthetic mimics of the extracellular matrix: how simple is complex enough? Ann Biomed Eng 43(3):489–500

Lareu RR, Subramhanya KH, Peng Y, Benny P, Chen C, Wang Z, Rajagopalan R, Raghunath M (2007a) Collagen matrix deposition is dramatically enhanced in vitro when crowded with charged macromolecules: the biological relevance of the excluded volume effect. FEBS Lett 581(14):2709–2714

Lareu RR, Arsianti I, Subramhanya HK, Yanxian P, Raghunath M (2007b) In vitro enhancement of collagen matrix formation and crosslinking for applications in tissue engineering: a preliminary study. Tissue Eng 13(2):385–391

Lee PJ, Choi AM (2003) Pathways of cell signaling in hyperoxia. Free Radic Biol Med 35(4):341–350

Lee S, Solow-Cordero DE, Kessler E, Takahara K, Greenspan DS (1997) Transforming growth factor-beta regulation of bone morphogenetic protein-1/procollagen C-proteinase and related proteins in fibrogenic cells and keratinocytes. J Biol Chem 272(30):19059–19066

Lee MH, Goralczyk AG, Kriszt R, Ang XM, Badowski C, Li Y, Summers SA, Toh SA, Yassin MS, Shabbir A, Sheppard A, Raghunath M (2016) ECM microenvironment unlocks brown adipogenic potential of adult human bone marrow-derived MSCs. Sci Rep 6:21173

Li Y, Kilian K (2015) Bridging the gap: from 2D cell culture to 3D microengineered extracellular matrices. Adv Healthc Mater 4(18):2780–2796
Lilla J, Stickens D, Werb Z (2002) Metalloproteases and adipogenesis: a weighty subject. Am J Pathol 160(5):1551–1554
Lin CQ, Bissell MJ (1993) Multi-faceted regulation of cell differentiation by extracellular matrix. FASEB J 7(9):737–743
Lodish H, Berk A, Zipursky S, Matsudaira P, Baltimore D, Darnell J (2000) Collagen: the fibrous proteins of the matrix. In: Molecular cell biology. W. H. Freeman, New York
Lutolf M, Hubbell J (2005) Synthetic biomaterials as instructive extracellular microenvironments for morphogenesis in tissue engineering. Nat Biotechnol 23(1):47–55
Magno V, Friedrichs J, Weber HM, Prewitz MC, Tsurkan MV, Werner C (2017) Macromolecular crowding for tailoring tissue-derived fibrillated matrices. Acta Biomater 55:109–119
Mather JP, Roberts PE (1998) The physical environment. In: Introduction to cell and tissue culture: theory and technique. Springer Science & Business Media, Boston, pp 25–41
McDowell L (2013) Vitamin history, the early years, 1st edn. Design eBook Publishing. http://firsteditiondesignpublishing.com/html/index.html
Minton AP (2001) The influence of macromolecular crowding and macromolecular confinement on biochemical reactions in physiological media. J Biol Chem 276(14):10577–10580
Mohyeldin A, Garzón-Muvdi T, Quiñones-Hinojosa A (2010) Oxygen in stem cell biology: a critical component of the stem cell niche. Cell Stem Cell 7(2):150–161
Mott JD, Thomas CL, Rosenbach MT, Takahara K, Greenspan DS, Banda MJ (2000) Post-translational proteolytic processing of procollagen C-terminal proteinase enhancer releases a metalloproteinase inhibitor. J Biol Chem 275(2):1384–1390
Mouw JK, Ou G, Weaver VM (2014) Extracellular matrix assembly: a multiscale deconstruction. Nat Rev Mol Cell Biol 15(12):771–785
Murad S, Grove D, Lindberg K, Reynolds G, Sivarajah A, Pinnell S (1981) Regulation of collagen synthesis by ascorbic acid. Proc Natl Acad Sci 78(5):2879–2882
Nakajima I, Aso H, Yamaguchi T, Ozutsumi K (1998) Adipose tissue extracellular matrix: newly organized by adipocytes during differentiation. Differentiation 63(4):193–200
Niles WD, Coassin PJ (2008) Cyclic olefin polymers: innovative materials for high-density multiwell plates. Assay Drug Dev Technol 6(4):577–590
Notbohm H, Mosler S, Bodo M, Yang C, Lehmann H, Bätge B, Müller PK (1992) Comparative study on the thermostability of collagen I of skin and bone: influence of posttranslational hydroxylation of prolyl and lysyl residues. J Protein Chem 11(6):635–643
Owens MR, Cimino CD (1982) Synthesis of fibronectin by the isolated perfused rat liver. Blood 59(6):1305–1309
Park D, Lim J, Park JY, Lee S-H (2015) Concise review: stem cell microenvironment on a chip: current technologies for tissue engineering and stem cell biology. Stem Cells Transl Med 4(11):1352–1368
Peng YX, Raghunath M (2010) Learning from Nature. Emulating macromolecular crowding to drive extracellular matrix enhancement for the creation of connective tissue in vitro. Source: Tissue Engineering, Book edited by: Daniel Eberli MD PhD, ISBN: 978-953-307-079-7, Publisher: INTECH, Publishing date: March 2010
Peng Y, Bocker MT, Holm J, Toh WS, Hughes CS, Kidwai F, Lajoie GA, Cao T, Lyko F, Raghunath M (2012) Human fibroblast matrices bio-assembled under macromolecular crowding support stable propagation of human embryonic stem cells. J Tissue Eng Regen Med 6(10):e74–e86
Peterkofsky B (1991) Ascorbate requirement for hydroxylation and secretion of procollagen: relationship to inhibition of collagen synthesis in scurvy. Am J Clin Nutr 54(6):1135S–1140S
Polacheck WJ, German AE, Mammoto A, Ingber DE, Kamm RD (2014) Mechanotransduction of fluid stresses governs 3D cell migration. Proc Natl Acad Sci 111(7):2447–2452
Prewitz MC, Stißel A, Friedrichs J, Träber N, Vogler S, Bornhäuser M, Werner C (2015) Extracellular matrix deposition of bone marrow stroma enhanced by macromolecular crowding. Biomaterials 73:60–69
Raghunath M, Bruckner P, Steinmann B (1994) Delayed triple helix formation of mutant collagen from patients with osteogenesis imperfecta. J Mol Biol 236(3):940–949

Raghunath M, Hopfner B, Aeschlimann D, Luthi U, Meuli M, Altermatt S, Gobet R, Bruckner-Tuderman L, Steinmann B (1996) Cross-linking of the dermo-epidermal junction of skin regenerating from keratinocyte autografts. Anchoring fibrils are a target for tissue transglutaminase. J Clin Invest 98(5):1174–1184

Raghunath M, Unsold C, Kubitscheck U, Bruckner-Tuderman L, Peters R, Meuli M (1998) The cutaneous microfibrillar apparatus contains latent transforming growth factor-beta binding protein-1 (LTBP-1) and is a repository for latent TGF-beta1. J Investig Dermatol 111(4):559–564

Raghunath M, Cankay R, Kubitscheck U, Fauteck JD, Mayne R, Aeschlimann D, Schlotzer-Schrehardt U (1999) Transglutaminase activity in the eye: cross-linking in epithelia and connective tissue structures. Invest Ophthalmol Vis Sci 40(12):2780–2787

Ramsey W, Hertl W, Nowlan E, Binkowski N (1984) Surface treatments and cell attachment. In Vitro 20(10):802–808

Rashid R, Lim NSJ, Chee SML, Png SN, Wohland T, Raghunath M (2014) Novel use for polyvinylpyrrolidone as a macromolecular crowder for enhanced extracellular matrix deposition and cell proliferation. Tissue Eng Part C Methods 20(12):994–1002

Ratcliffe A, Niklason LE (2002) Bioreactors and bioprocessing for tissue engineering. Ann N Y Acad Sci 961(1):210–215

Rollason G, Davies JE, Sefton MV (1993) Preliminary report on cell culture on a thermally reversible copolymer. Biomaterials 14(2):153–155

Royce PM, Steinmann B (2003) Connective tissue and its heritable disorders: molecular, genetic, and medical aspects. Wiley, New York

Ruoslahti E (1996) RGD and other recognition sequences for integrins. Annu Rev Cell Dev Biol 12(1):697–715

Satyam A, Kumar P, Fan X, Gorelov A, Rochev Y, Joshi L, Peinado H, Lyden D, Thomas B, Rodriguez B, Raghunath M, Pandit A, Zeugolis D (2014) Macromolecular crowding meets tissue engineering by self-assembly: a paradigm shift in regenerative medicine. Adv Mater 26(19):3024–3034

Satyam A, Kumar P, Cigognini D, Pandit A, Zeugolis DI (2016) Low, but not too low, oxygen tension and macromolecular crowding accelerate extracellular matrix deposition in human dermal fibroblast culture. Acta Biomater 44:221–231

Sbarbati A, Zancanaro C, Cigolini M, Cinti S (1987) Brown adipose tissue: a scanning electron microscopic study of tissue and cultured adipocytes. Acta Anat (Basel) 128(1):84–88

Selvarajan S, Lund LR, Takeuchi T, Craik CS, Werb Z (2001) A plasma kallikrein-dependent plasminogen cascade required for adipocyte differentiation. Nat Cell Biol 3(3):267–275

Sholoqu N, Szegezdi E, Lowery A, Kerin M, Pandit A, Zeugolis D (2016) Recreating complex pathophysiologies in vitro with extracellular matrix surrogates for anticancer therapeutics screening. Drug Discov Today 21(9):1521–1531

Shoulders MD, Raines RT (2009) Collagen structure and stability. Annu Rev Biochem 78:929–958

Stenman S, Vaheri A (1978) Distribution of a major connective tissue protein, fibronectin, in normal human tissues. J Exp Med 147(4):1054–1064

Taipale J, Saharinen J, Hedman K, Keski-Oja J (1996) Latent transforming growth factor-beta 1 and its binding protein are components of extracellular matrix microfibrils. J Histochem Cytochem 44(8):875–889

Theocharis AD, Skandalis SS, Gialeli C, Karamanos NK (2016) Extracellular matrix structure. Adv Drug Deliv Rev 97:4–27

Trackman PC (2005) Diverse biological functions of extracellular collagen processing enzymes. J Cell Biochem 96(5):927–937

Van der Valk J, Mellor D, Brands R, Fischer R, Gruber F, Gstraunthaler G, Hellebrekers L, Hyllner J, Jonker F, Prieto P (2004) The humane collection of fetal bovine serum and possibilities for serum-free cell and tissue culture. Toxicol In Vitro 18(1):1–12

Van der Valk J, Brunner D, De Smet K, Svenningsen ÅF, Honegger P, Knudsen LE, Lindl T, Noraberg J, Price A, Scarino M (2010) Optimization of chemically defined cell culture media – replacing fetal bovine serum in mammalian in vitro methods. Toxicol In Vitro 24(4):1053–1063

von Recum H, Kikuchi A, Okuhara M, Sakurai Y, Okano T, Kim SW (1998) Retinal pigmented epithelium cultures on thermally responsive polymer porous substrates. J Biomat Sci Polym Ed 9(11):1241–1253

Wang Z, Griffin M (2012) TG2, a novel extracellular protein with multiple functions. Amino Acids 42(2–3):939–949

Werner J, Buse M (1988) Temperature profiles with respect to inhomogeneity and geometry of the human body. J Appl Physiol 65(3):1110–1118

Woolridge E, Turchi SL, Edwards JR (1986) The peroxidase-glucose oxidase enzyme system in the undergraduate laboratory. Biochem Educ 14(2):82–83

Yamato M, Okano T (2004) Cell sheet engineering. Mater Today 7(5):42–47

Yamato M, Akiyama Y, Kobayashi J, Yang J, Kikuchi A, Okano T (2007) Temperature-responsive cell culture surfaces for regenerative medicine with cell sheet engineering. Prog Polym Sci 32(8):1123–1133

Yamauchi M, Sricholpech M (2012) Lysine post-translational modifications of collagen. Essays Biochem 52:113–133

Yang J, Yamato M, Kohno C, Nishimoto A, Sekine H, Fukai F, Okano T (2005) Cell sheet engineering: recreating tissues without biodegradable scaffolds. Biomaterials 26(33):6415–6422

Zeiger AS, Loe FC, Li R, Raghunath M, Van Vliet KJ (2012) Macromolecular crowding directs extracellular matrix organization and mesenchymal stem cell behavior. PLoS One 7(5):e37904

Zeugolis DI, Panengad PP, Yew ES, Sheppard C, Phan TT, Raghunath M (2010) An in situ and in vitro investigation for the transglutaminase potential in tissue engineering. J Biomed Mater Res A 92(4):1310–1320

Zheng X, Baker H, Hancock WS, Fawaz F, McCaman M, Pungor E (2006) Proteomic analysis for the assessment of different lots of fetal bovine serum as a raw material for cell culture. Part IV. Application of proteomics to the manufacture of biological drugs. Biotechnol Prog 22(5):1294–1300

Zhou H-X (2008) Effect of mixed macromolecular crowding agents on protein folding. Proteins 72(4):1109–1113

Zhou HX, Rivas G, Minton AP (2008) Macromolecular crowding and confinement: biochemical, biophysical, and potential physiological consequences. Annu Rev Biophys 37:375–397