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A 3D Alzheimer's disease culture model and the induction of P21-activated kinase mediated sensing in iPSC derived neurons

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ABSTRACT

The recent progress in stem cell techniques has broadened the horizon for *in vitro* disease modeling. For desired *in vivo* like phenotypes, not only correct cell type specification will be critical, the microenvironmental context will be essential to achieve relevant responses. We demonstrate how a three dimensional (3D) culture of stem cell derived neurons can induce *in vivo* like responses related to Alzheimer's disease, not recapitulated with conventional 2D cultures. To acquire a neural population of cells we differentiated neurons from neuroepithelial stem cells, derived from induced pluripotent stem cells. p21-activated kinase mediated sensing of Aβ oligomers was only possible in the 3D environment. Further, the 3D phenotype showed clear effects on F-actin associated proteins, connected to the disease processes. We propose that the 3D *in vitro* model has higher resemblance to the AD pathology than conventional 2D cultures and could be used in further studies of the disease.

1. Introduction

Modeling of neural diseases is challenging, but essential to provide insights into disease mechanisms and strategies for treatments. Animals can constitute powerful models of the nervous system. However, their high cost and ethical complexity, as well as the poor translation between most animals and humans raise the demand for human *in vitro* models. Derivation of specific neural cells from induced pluripotent stem (iPS) cells [1], has made it possible to create *in vitro* models that recapitulate some neural disease phenotypes. The technique offers new possibilities to study neural diseases with unknown genetic causes, as well as looking into basic cellular mechanisms and studies, such as a whole transcriptome sequencing. Alzheimer's disease (AD) is one example where improved *in vitro* models raise the hope to find insights into disease mechanisms and possible treatment strategies. Currently 35 million persons world-wide are afflicted with AD and the numbers are expected to increase with an aging population [2]. A number of recent reports based on iPS cell derivation and subsequent differentiation into neurons from both familial and sporadic AD have been able to recapitulate certain disease characteristic in the dish [3–6].

The environmental context can fundamentally change the state and fate of a specific cell [7,8]. Mechanical signals are critical for example for nociceptive neurons, as indicated in a seminal finding that the stiffness of materials surrounding mesenchymal stem cells is sufficient to direct them to the neuronal lineage [9]. In addition, neuronal maturation has been augmented on softer substrates [10]. The transfer of physical stimuli, such as the mechanical properties of a 3D matrix, to chemical signals in a cell is referred to as mechanotransduction. Mechanotransduction in neural cells have been observed as changes in e.g. neurite growth, cell spreading, survival and cytoskeleton composition [11]. Numerous *in vitro* approaches are mimicking several spatial-temporal cell extrinsic stimuli, often in the form of the 3D tissue culture, as reviewed by Tibbitt and Anseth [8]. *In vitro* models of AD are commonly based on neuronal-like cell lines such as neuroblastoma lines or primary animal derived cultures. Recent reports demonstrate improved resemblance of the disease in 3D spheroid cultures compared to 2D cultures [12,13]. Due to the self-assembling nature of spheroid

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cultures they are difficult to control and the cell microenvironment differs significantly depending on the distance to the spheroid surface. Moreover, the possibilities of high resolution microscopy studies is limited [12,13]. A 3D hydrogel culture offers a suitable stiffness for neural cells and higher degree of control of cellular context. RADA-16 is a synthetic peptide that upon addition of cations can self-assemble to nanofibrous hydrogel [14]. RADA-16, from now on called self-assembling peptide (SAP) matrix, has been proven to support long-term culture of neural cells derived from human embryonic stem cells [15].

P21-activated kinases (PAKs) have been shown to be critical to mechanotransduction pathways [16]. These small GTPase activated kinases provide a link between mechanical forces, cytoskeleton dynamics, and neuronal morphology [17]. PAK1 and PAK3 have been mostly studied for their impact on neuronal function, specifically in the developing forebrain [17]. Interestingly, PAKs have also been linked to neural diseases, including Alzheimer's disease and X-linked mental retardation [18]. Both PAK1 and PAK3 levels have been shown to be reduced in late stage AD patient brains, on protein level and even more on activated protein level (pPAK) [19]. In AD pathology, as well as in mouse models and in cultured hippocampal neurons stimulated with Aβ oligomers, an aberrant translocation of activated PAK from the cytosol to the cell membrane is evident [20]. PAK regulates F-actin cytoskeleton and dendritic spine dynamics through activation of LIM-kinase. LIM-kinase in turn dephosphorylates the actin binding protein cofilin, resulting in actin depolymerization activity. Cofilin levels has moreover shown a reciprocal relation to pPAK in AD brains, where increased cofilin staining has been corresponding to decreased diffuse pPAK staining [20]. Intracellular inclusion bodies (Hirano bodies) of cofilin and short actin rods decorated by other proteins are pathological features of AD [21]. Drebrin, which is an actin stabilizing protein important for spine morphogenesis, has been observed to be significantly reduced in AD brain (70–95%) [22]. It has been shown that there is a reciprocal relationship between cofilin and drebrin levels in AD patient samples and *in vitro* experiments on cultured neurons suggest that cofilin competes for drebrin binding sites on actin filaments [20]. The same study also showed that overexpression of active PAK could rescue Aβ induced loss of drebrin. Together, these observations suggest that there is a cross-talk between mechanotransduction pathways and Aβ induced pathology and that PAK is a central mediator of this response.

It is still a challenge to reproducibly derive pure cell cultures, such as neuronal cell cultures from iPS cells or human embryonic stem cells. One route to enhance neuronal differentiation efficiency is to use neuroepithelial like stem cells, It-NES cells, neural progenitors that can be stably expanded long-term in culture [23]. It-NES cells are highly neurogenic and repeatedly give >90% neurons and lower amounts of glial cells upon differentiation. In later stage Alzheimer's disease diverse atrophy of the brain can be observed, whereas early on the disease progression affects the neurons of the cortex, especially in the hippocampus and cholinergic neurons of the forebrain. To acquire a mixed population of neurons, we applied trophic factors that can support the induction and survival of cholinergic neurons, while still giving gabaergic, glutamatergic and dopaminergic neurons.

Here, we present a 3D cell culture that has the potential to mimic the 3D neuronal environment, with the ambition to use it as a model of Alzheimer's disease. It could also be applied to study other neural diseases, especially those linked to modulated PAK signaling. We took the route of using a self-assembling peptide hydrogel to provide a 3D support structure. Moreover, the human iPS cells were used via the neuroepithelial stem cell state to acquire a cell model based on a neural population with a high percentage of neurons and a low number of glial cells.

2. Materials and methods

2.1. Cell culture

Human iPSC derived neuroepithelial stem cells (It-NES) line AF22 were cultured according to previously published protocol [23]. Briefly, It-NES cells were maintained in the DMEM/F12 GlutaMAX (Gibco 31331) supplemented with 10 ng/mL rhFGF-basic (R&D systems Cat. 233-FB), 10 ng/mL rhEGF (R&D systems Cat. 236-EG), B-27 supplement (1:1000, Gibco, Cat. 17504-044) and N-2 supplement (1:100, Gibco, Cat. 17502-048) and 100 Units of Penicillin/Streptomycin (Gibco, Cat. 15140). It-NES cells were passaged at a ratio of 1:3 every second to third day using TrypLE Express (Gibco, Cat. 12604) and Defined trypsin inhibitor (Gibco, Cat. R-007-100). It-NES cells were cultured on double-coated plates with 20 μg/mL of Poly-L-Ornithine (Sigma, Cat. P4957) and 2 μg/mL of Laminin (Sigma, Cat. L2020). Differentiation was induced from It-NES cells through sequential treatments of growth factors according to the following schedule. Confluent It-NES cells were passaged at ratio of 1:3 into DMEM/F12 GlutaMAX and Neurobasal media (Gibco 21103) (50:50), supplemented with 100 nM of Sonic Hedgehog agonist Ag1.3 (Curis Inc.), 10 ng/mL Fgf8 (R&D Systems Cat. 423-F8/CF), N-2 supplement (1:100), B-27 supplement without Vitamin A (1:100, Gibco, Cat. 12587-010) and Penicillin/Streptomycin (1:100) for 3 full days with half medium change next day. After 3 days, cells were passaged at ratio of 1:5 into neuron medium 1 containing DMEM/F12 GlutaMAX and Neurobasal media (30:70) supplemented with 50 ng/mL rhβ-NGF (R&D systems Cat. 256-GF/CF), N-2 supplement (1:100), B-27 supplement without vitamin A (1:50) and Penicillin/Streptomycin (1:100) for 5 full days with half medium change every second day. At this stage, for the first 3 days, cells were also exposed to 10 ng/mL BMP9 (R&D Systems Cat. 3209-BP010CF), and thereafter the cells were only cultured into neuron medium 1. Cells were then cultured in the optimized neuron medium 2 containing Neurobasal medium supplemented with 50 ng/mL rhβ-NGF, N-2 supplement (1:100), B-27 supplement without vitamin A (1:100), GlutaMAX (1:200, Gibco, Cat. 35050), L-Glutamine (1:200, Gibco, Cat. 25030) and Penicillin/Streptomycin (1:100) for 2 weeks with half media change every second day. At this stage of day 4 and day 5, the medium was also supplemented with 1 μM arabinosylcytosine (AraC, Sigma) to eliminate the growth of bFGF-responsive cells.

Spontaneous neuronal differentiation was also induced from It-NES cells by removal of rhFGF-basic and rhEGF. Fully confluent It-NES cells were passaged at ratio of 1:3 into PDL-Laminin coated flask in the DMEM/F12 GlutaMAX supplemented with 1% N-2 supplement, 0.1% B-27 supplement and 1% penicillin/Streptomycin for 7 days with half medium change every second day; and then cells were moved to DMEM/F12 GlutaMAX and Neurobasal media (50:50) supplemented with 0.5% N-2 supplement, 1% B-27 supplement, 0.5% Glutamax, 1% penicillin/Streptomycin for 9 days with half media change every second day.

2.2. Immunofluorescence

Cultures were fixed at 4% paraformaldehyde (Sigma) in phosphate-buffered saline (PBS) for 30 min at room temperature. Next, fixed cells were permeabilized in PBS with 0.1% Triton-X 100 (TX-100). The following primary antibodies were used for immunofluorescence experiments: chicken anti-ChAT (Aves, 1:1000), rabbit anti-Tuj1 (Covance, 1:1000), goat anti-TrkA (R&D systems, 1:1000), p75NTR (Abcam, 1:1000), Nestin (Millipore, 1:1000), rabbit anti-pPAK (Invitrogen, 1:500), rabbit anti-cofilin (Millipore, 1:500) and mouse anti-drebrin (Abcam 1:300). The secondary antibodies were anti-chicken, rabbit, mouse or goat IgG conjugated with Fluorescein (Aves), or Alexa-488. −546, or −647 (Invitrogen). 4′, 6-diamidino-2-phenylindole (DAPI) was used at dilution of 1:1000 for nuclei staining. Alexa Fluor-488 Phalloidin (Invitrogen) was used at dilution of 1:400. Samples were imaged on a Zeiss 510 LSM META confocal microscope using a 20× air objective. Image acquisition was carried out at 1024 × 1024 pix and all comparisons between 2D and 3D cultures were carried out with the same laser intensity, gain settings and optical slice thickness. Image processing was carried out in Photoshop and image analysis was done in Photoshop or Cellprofiler. Statistical data comparing 2D and 3D SAP matrix cultures were carried out in Prism (GraphPad) using *t*-tests. Significance levels were $^{***}p < 0.001$ and $^{**}p < 0.01$. Number of independent replicates was three in all cases except for cofilin were two replicates were used. All data for other immunofluorescence stainings were repeated at least three times and representative images were chosen for display.

2.2.1. Aβ42 oligomer preparation

Biotinylated Aβ42 (Bachem, Cat. H5642), was fully dissolved at 0.5 mg/mL in hexafluor-2-propanole (HFIP, Sigma). 10 μL of HFIP Aβ42 solution was dispensed into siliconized Snap-Cap microtube (Sigma), put in a desiccator to completely evaporate HFIP and thereafter stored at −80 °C. Monomer and oligomer solutions were prepared fresh for each cell stimulation experiment at 100 nM, for 24 h. For oligomer formation, the stock was dissolved in 10 μL of DMSO (to 105 μM), and further diluted in PBS to 1 μM, incubated for 3 h at room temperature. For monomer formation, 0.1% Tween-20 was added to inhibit the oligomerization process, 1:1000 dilution was used when added to cell cultures. To view the deposition of Aβ42 oligomers on cells, Streptavidin Alexa Fluor-488 (Invitrogen, Cat.S32354, dilution 1:1000) was used. To test the degree of oligomerization of Aβ42, oligomers were cross-linked with so-called PICUP reaction. Briefly 18 μL 100 nM of Aβ sample is mixed with 1 μL

Ammonium persulfate, APS, (20 mM) and 1 μL Tris (2,2′-bipyridyl)dichlororuthenium(II) hexahydrate Ru(Bpy) (1 mM). The sample is irradiated for 1 min with the lamp in the overhead projector. Finally evaluation of oligomerization was done with western blot according to standard protocols and Streptavidin-HRP.

2.3. qPCR

RNA was isolated using RNeasy kit (Qiagen), and cDNA was made with High capacity cDNA reverse transcription kit (ABI, part nr. 4368814), and specific genes were amplified using Fast SYBR Green Master mix kit (ABI, part nr. 4385612). Standard protocols were used to run the 7500 Fast Real-Time PCR System (Applied Biosystems). Results are an average of three independent experiments. Primers are listed in Supplementary Information.

2.4. PuraMatrix (PM) hydrogel preparation

PM peptide hydrogel was purchased from BD (Cat. 354250). In this study, 0.2% PM hydrogel was used according to manufacturer's instruction, and 10 μg/mL laminin was incorporated as a modification for It-NES cell and neuronal differentiation. The volume of the hydrogel was 0.2 mL and the seeding density of the cells was 625,000 cells/mL. The cells were seeded to the hydrogel before the final gelation was finished, normally within 20–30 min.

3. Results

3.1. Survial of It-NES cells and differentiated neurons in SAP matrix

To assess the potential of the SAP matrix for culture of human neural iPS derived cells, we first seeded 3D SAP scaffolds with It-NES cells and cultured them for 2 days. The thickness of the gel was around 2 mm, slightly thicker in the edges due to liquid meniscus effect. Live/dead cell fluorescence imaging showed negligible dead cell staining in 3D culture of It-NES cells, comparable to the numbers of dead cells in 2D cell culture (Fig. 1a, b). Further, Nestin, a neural progenitor marker, showed clear positive staining in both 2D and 3D cultures (Fig. 1c, d). Viable It-NES cells, stained with calcein-AM or nestin, occasionally formed neural-rosette-like clusters in 2D and 3D cultures, but were more frequent and more evenly distributed in the culture area/volume in 3D cultures (Fig. 1). It-NES cells cultured in the 3D matrix showed similar individual morphology to their counterparts on 2D, although bright field microscopy is difficult to apply in 3D (Fig. 1e, f). SAP matrix is highly water containing and diffusion of low molecular species, e.g. gases such as O_2 and CO_2 is virtually unchanged compared to cell medium, protein diffusion has however been shown to be slightly impacted [24]. Long term cultures in SAP matrix are technically challenging due to the very low stiffness of the material. Multiple media changes will eventually lead to disruption of the gel. However, cultures for over four weeks have been reported [15], but preferably culture times should be kept under two weeks. Therefore our route was to first differentiate NES cells in 2D and then sub-culture the differentiated neurons in the 3D matrix (Fig. S1) for two days. To test the impact of 3D matrix on the morphology of differentiated neurons, we used light microscopy, which showed the presence of complex, branched neuronal structures both in 2D and 3D (Fig. 1g, h, Fig. S2).

Light microscopy revealed that some cells remained at the top of the 3D matrix, whereas other cells went through the matrix and were distributed vertically in different layers.

3.2. Characterization of differentiated neurons

We sequentially applied extrinsic factors; Shh, FGF8, BMP9 and NGF to differentiating It-NES cells. These factors can support commitment and survival of cholinergic neurons in the forebrain. (Schematic schedule in Fig. S1). Neuronal differentiation in monolayer culture was evident with light microscopy (Fig. 1g, Figs. S1, S2). 3D cultures were carried out as previously described, with differentiation in 2D cultures and subculturing in 3D SAP matrix for two days prior to analysis. Immunocytochemical staining revealed an absolute majority of cells (>90%) in 2D and 3D culture with strong Tuj1 staining, indicative of a neuronal cell type (Fig. S3). Moreover cells were largely double positive for GABA and Tuj1 (Fig. S3), consistent with a previous report showing that spontaneous differentiation of It-NES cells in absence of growth factors gives predominantly Gabaergic neurons [23]. A low percentage (<10%) of glial fibrillary acidic protein (GFAP) positive glial cells were observed in 2D as well in 3D SAP matrix culture (data not shown). Immunocytochemical staining of differentiations with sequential exposure to the above mentioned factors also showed colonies with expression of choline acetyl transferase (ChAT) (Figs. S5a, S6a) in both conventional 2D culture and SAP matrix 3D culture, indicative of a cholinergic neuronal subtype. ChAT positive cells were not observed in the spontaneous differentiation. In addition to the ChAT staining,

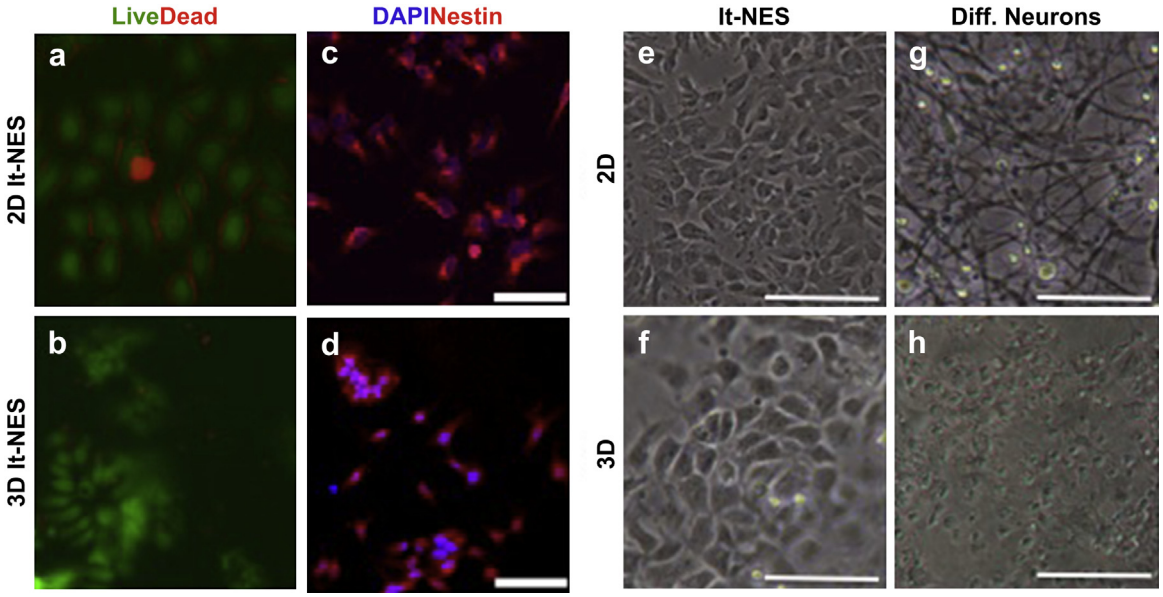

Fig. 1. Comparison between conventional 2D culture and 3D culture in SAP matrix, a–b) illustrating a comparable number of dead NES cells in a Live/Dead assay, c–d) Nestin (neural stem cell marker) staining demonstrating that the neural stem cell state is maintained and e–f) bright field images of NES and differentiated neurons. It-NES cells and differentiated neurons were cultured in the SAP matrix for 2 days.

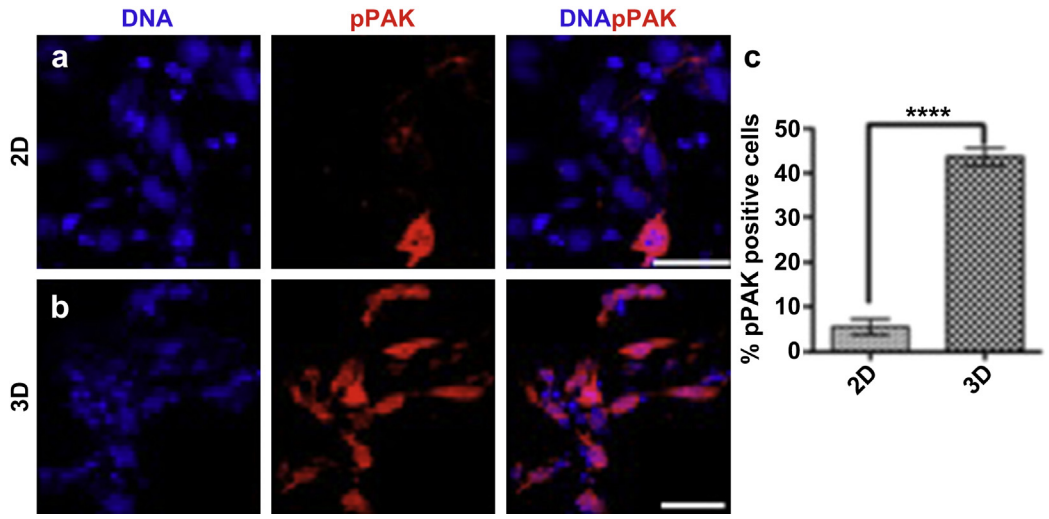

Fig. 2. Activation of pPAK a–b) The mechanotransduction mediator PAK shows higher activation levels in 3D SAP matrix compared to 2D culture, quantified in c). The scale bars represent 50 μm. All immunocytochemical images were obtained with an inverted confocal microscope, imaging cells that penetrated into the matrix, which are in a 3D microenvironment. 2D and 3D images were acquired using same optical slice thickness.

the presence of cholinergic neurons in the culture is supported by the finding of colonies positive for the neurotrophin receptors TrkA and p75 (Figs. S5, S6). Moreover qPCR evaluation revealed upregulation of mRNA of markers of forebrain cholinergic neurons transcription factors, Lhx8 (LIM homeobox protein 8) and Gbx1 (Gastrulation and brain-specific homeobox protein 1), as well as of choline acetyl transferase (ChAT) and acetyl choline esterase AChE and all four neurotrophin receptors, p75NTR, TrkA, TrkB and TrkC (Fig. S4). Together these data shows that the applied protocol gives mixed subtypes of predominantly neuronal cells.

The differentiated neurons in the 3D SAP matrix were morphologically resembling the cells in 2D culture, but with neurites growing in a 3D manner. Smaller differences in distribution in the markers mentioned above could be seen in 3D SAP matrix cultures compared to 2D (Figs. S5, S6)

3.3. SAP matrix effect on pPAK and cytoskeleton-associated proteins

To evaluate the effect of the SAP matrix on the activation profile of PAK, we used immunocytochemistry to examine the expression of activated form of PAK (pPAK). Interestingly, the levels of pPAK in 3D SAP culture of differentiated neurons were significantly higher than those in 2D (Fig. 2a–c, Fig. S7). Since cofilin is also involved in the regulation of cytoskeletal dynamics by dissembling the actin filaments, we next tested the effect of 3D culture on the expression of cofilin. Immunocytochemical analysis revealed that differentiated neurons cultured in 2D demonstrated higher expression level of cofilin compared to 3D SAP cultures (Figs. 3 and 4). The low cofilin expression in 3D SAP matrix is consistent with the observation that pPAK levels are higher in 3D than in 2D cultures. For F-actin, however, we could not detect a significant difference in expression in 2D and 3D cultures (Figs. 4 and 5). In 3D SAP matrix culture, the reciprocal relationship between low cofilin levels and high drebrin levels, demonstrated in AD, could be observed (Fig. 4). In contrast, 2D cultures mostly failed to recapitulate this relationship. In embryonic cortical neurons drebrin has been shown to localize with F-actin at neuronal growth cones [25]. We studied the localization of drebrin compared to F-actin both in 2D and 3D culture of differentiated neurons (Fig. 5). Drebrin was observed in neurites and cell soma both in 2D and 3D culture. However the 3D

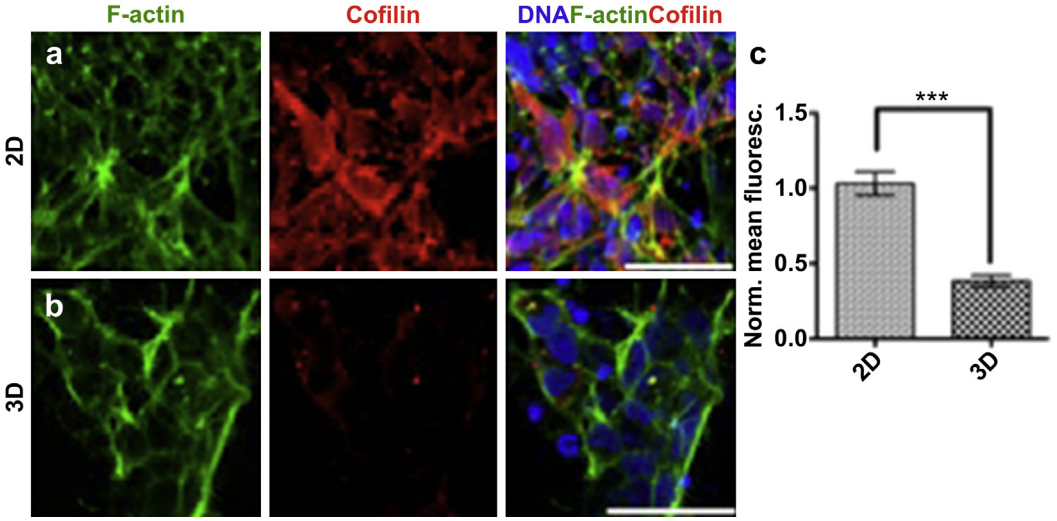

Fig. 3. Immunocytochemistry of F-actin and cofilin. a–b) Cofilin is higher expressed in 2D cultures compared to 3D SAP matrix, quantified in c), whereas no significant difference is observed in F-actin (phalloidin) staining. The scale bars represent 50 μm.

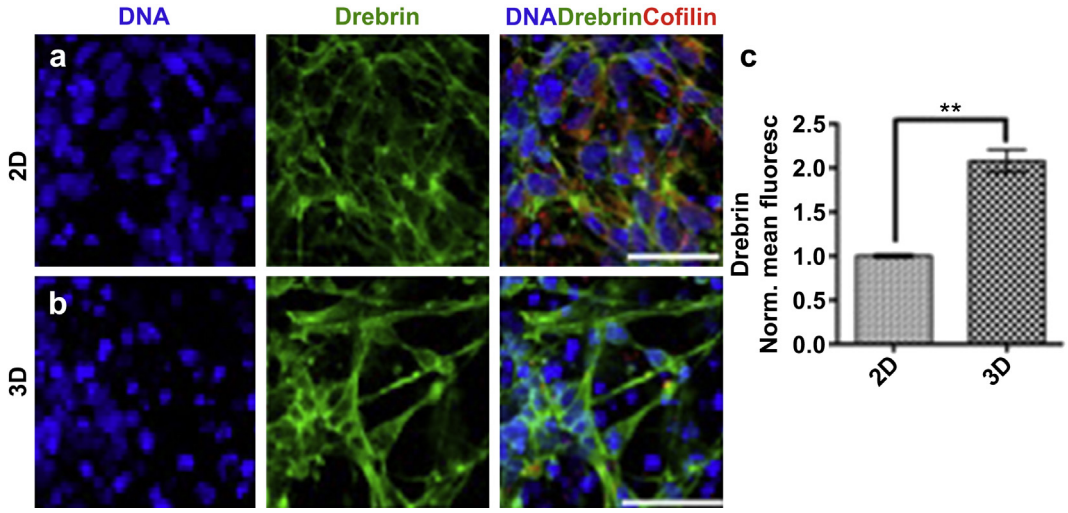

Fig. 4. Levels of cytoskeleton-associated proteins drebrin and cofilin. a–b) Cytoskeleton regulator drebrin is lower expressed in 2D compared to 3D SAP matrix, quantified in c), whereas the relation is the opposite for cofilin. The scale bars represent 50 μm.

cultures indicated higher levels of co-localization of drebrin and F-actin both at cell soma and in neurites (Fig. 5 and Movies S1 and S2). In contrast, in 2D cultures drebrin and F-actin only displayed low co-localization in some soma regions (Fig. 5 and Supplementary movies).

Supplementary video related to this article can be found at http://dx.doi.org/10.1016/j.biomaterials.2013.11.028.

3.4. Evaluation of response to Aβ oligomers

Aβ oligomer production has been implicated to be the direct cause of the pathological symptoms of Alzheimer's disease [26]. To evaluate if the 3D culture model could be used to investigate the molecular basis that accounts for the defects caused by Aβ oligomers, we treated differentiated neurons with 100 nM Aβ oligomer. We generated Aβ oligomers fresh for each experiment, due to their high tendency to aggregate into larger assemblies, and evaluated with western blot (Fig. 6a). Our protocol yielded mostly Aβ in form of 12-mer oligomers, which upon further assembly would aggregate to form amyloid fibrils. To facilitate the observation of localization of Aβ oligomers, we used a biotin tagged peptide, and upon staining with avidin-Alexa 488, a similar distribution pattern on cells in 2D and 3D environments was revealed (Fig. 6b).

Next, we examined how Aβ oligomer treatments affect the pPAK sub-cellular localization, given the reported relation between pPAK distribution and AD pathology [18]. Differentiated neurons were treated with 100 nM Aβ oligomers for 24 h, followed by immunocytochemistry. In 3D SAP matrix culture Aβ oligomer treatment caused depletion of activated form of PAK in both cytosolic and nucleus regions, and redistributed the pPAK to submembranous regions, forming a thin circular distribution of the protein (Fig. 7). In some cells, fragmental pPAK staining was observed (Fig. 7). This aberrant translocation resembles what has been observed both in AD patient brains and mouse models of AD [19,20,27], although

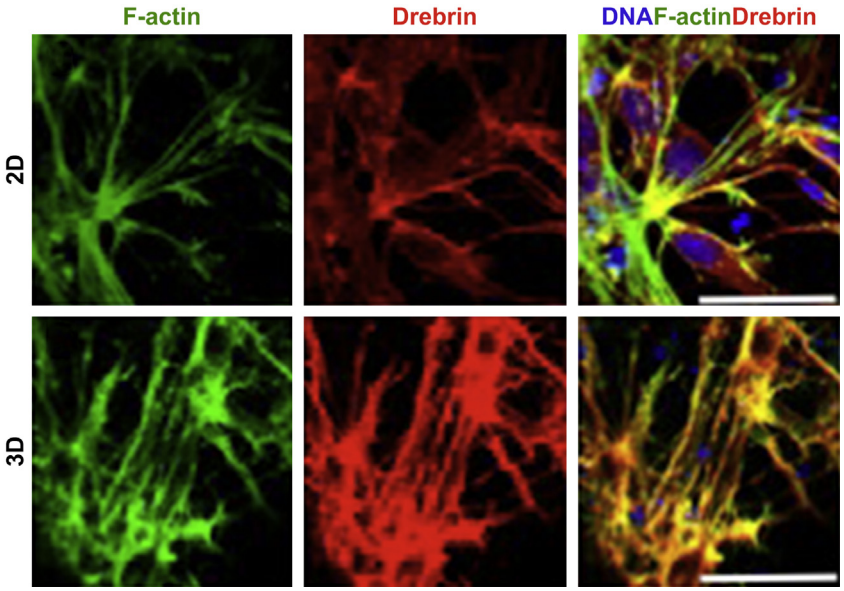

Fig. 5. Staining of F-actin and drebrin. a–b) Co-staining of F-actin (phalloidin) and drebrin illustrates higher degree of co-localization for cells in the 3D SAP matrix. The scale bars represent 50 μm.

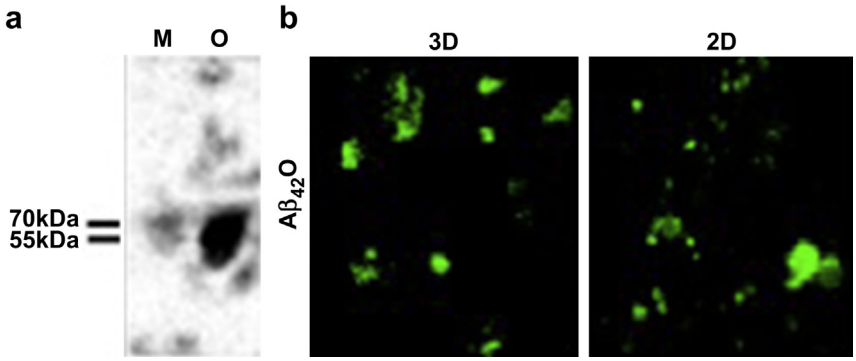

Fig. 6. Aβ oligomer characterization and cell exposure a) WB of Aβ monomers and oligomers b) FITC-avidin staining of the oligomers of biotinylated Aβ42 on differentiated neurons showing no differences in distribution of cells between 3D SAP matrix and conventional 2D culture.

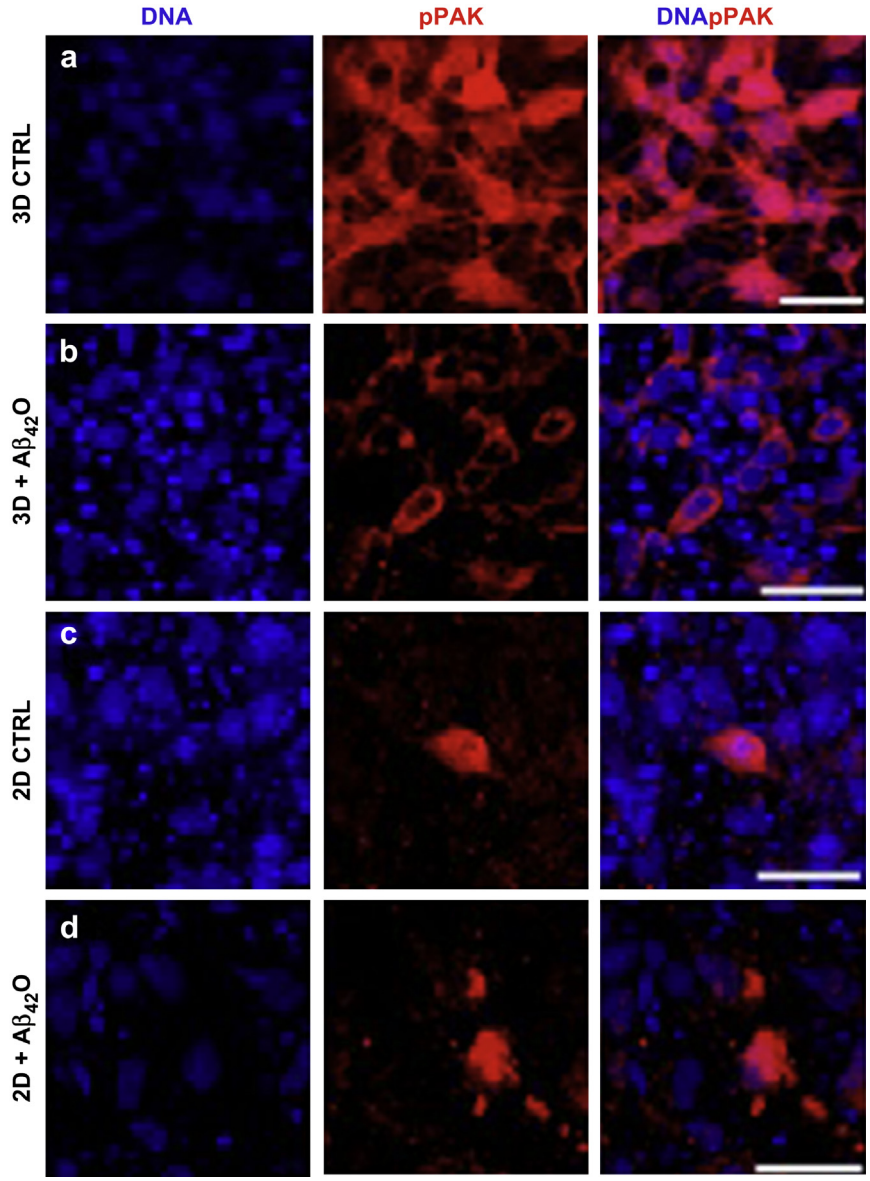

Fig. 7. pPAK immunocytochemistry after Aβ oligomer treatment. a) 3D SAP matrix control show pPAK staining distributed throughout the cells where b) Aβ oligomer treatment resulted in a redistribution of the activated protein. In conventional 2D culture c–d) this redistribution could not be seen in the low number of cells with positive staining for pPAK. The scale bars represent 50 μm.

severe AD is more associated with fragmented staining of pPAK associated to cell membrane. In contrast, Aβ oligomer treatment of 2D-cultured differentiated neurons did not give notable changes in pPAK distribution compared to controls, both cases having low expression of pPAK (Fig. 7). Drebrin was also affected by the treatment with Aβ oligomer on the 3D cultures (Fig. 8), but not on comparable 2D cultures (results not shown). Drebrin was significantly decreased from the cytosolic regions, and markedly reduced from neurites regions. However, in submembranous regions a clear drebrin staining could still be observed after oligomer treatment. The reduction of drebrin in the mentioned regions upon Aβ oligomer treatment is in line with previous studies on the relation between drebrin and PAK in AD. The observation that both pPAK and drebrin relocalize in a similar distribution pattern after Aβ stimulation, does not necessarily imply functional interaction between the two proteins, but would need further studies.

4. Discussion

This study has the aim to improve cell culture models of Alzheimer's disease to recapitulate some of the complex processes that results in this prevalent and currently untreatable condition. Our route was neuronal differentiation of human iPS cells and subculturing in a soft self-assembling peptide hydrogel, RADA-16. RADA-16 has been previously assessed for survival of human neurons derived form embryonic stem cells [15]. As previously reported we could confirm that the SAP matrix is too acidic for direct mixing with human neuronal cells (results not shown). We took the route of initiating gel formation with a buffer and seeded the cells before gelation was complete, which gave a distribution of cells throughout the matrix. This procedure resulted in very good cell survival and neurite outgrowth, evaluated with Live/Dead and immunocytochemical staining respectively.

Cell models of AD are often based on human cancer derived cell lines or animal derived primary culture. The translation of both these sources to the human disease progression has been questioned [28]. Our approach was to derive human neuronal cells from pluripotent stem cells. To obtain a pure neural population, without the need for cell sorting, we used a neuroepithelial stem cell line derived from human iPS cells [23]. It-NES cells have been reported to differentiate into a mixed population of hind- and midbrain neuronal subtypes and to a low percentage of glial cells. Since AD with progression leads to general neurodegeneration we applied a protocol to get a neuronal population that apart from hind- and midbrain neurons also included cells with forebrain character. In this study iPS cells from healthy individuals were used. However in the future studies, the patient specific iPS cells could be used to model neural diseases.

In vivo cell function is truly affected by the three-dimensional organization of tissue. *In vitro* 3D structure giving a suitable physical and chemical environment can recapitulate some aspects of this natural niche for neurons and for example promote physiological neuronal properties [11]. It is easy to relate this to the seminal study by Cukierman et al., where *in vivo* like adhesion patterns could be induced *in vitro* by proper combination of 3D architecture, molecular composition and appropriate mechanical forces [29]. In this study we observed that general neuronal markers and certain cell surface receptors such as TrkA and p75, had similar expression levels in 2D and 3D *in vitro* environment. However when focusing on regulators of mechanotransduction and cytoskeletal dynamics, we found striking differences, which interestingly have clear relations to AD. We identified that the activity of PAK responds to the SAP 3D matrix, which is also reflected in cytoskeleton-associated proteins with low cofilin and high drebrin expression. In the 3D SAP matrix culture we could moreover observe a higher degree of co-localization of F-actin and drebrin, which is normally seen in growth cones of neurons [25]. In conventional 2D cultures most neurons have significantly lower levels of activated PAK. We speculate that this might be due to that in the SAP matrix the differentiated neurons are immersed in a mechanical surrounding with stiffness of a few Pascal, much more resembling brain tissue than a traditional 2D culture [11,30]. Morphology is key to function of neurons and the local environment is key to form a proper morphology. The dynamics of assembly and disassembly of filamentous actin is the molecular basis behind the formation of appropriate neuronal morphology. This dynamic process is regulated by a number of actin-associated proteins, in which drebrin and cofilin play an important role. In

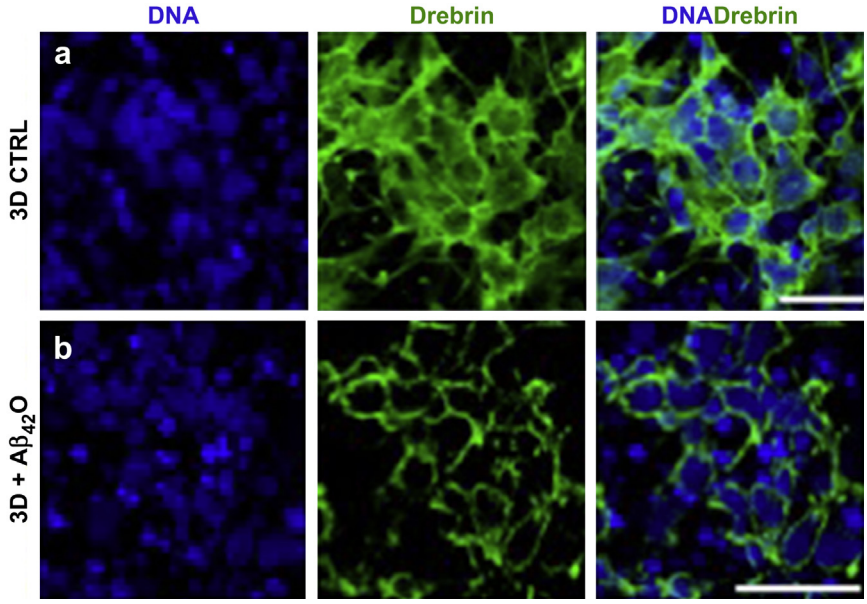

Fig. 8. Drebrin and pPAK immunocytochemistry after Aβ oligomer treatment. a–b) Drebrin also show a clear re-localization in 3D SAP matrix upon Aβ oligomer stimulation. The scale bars represent 50 μm.

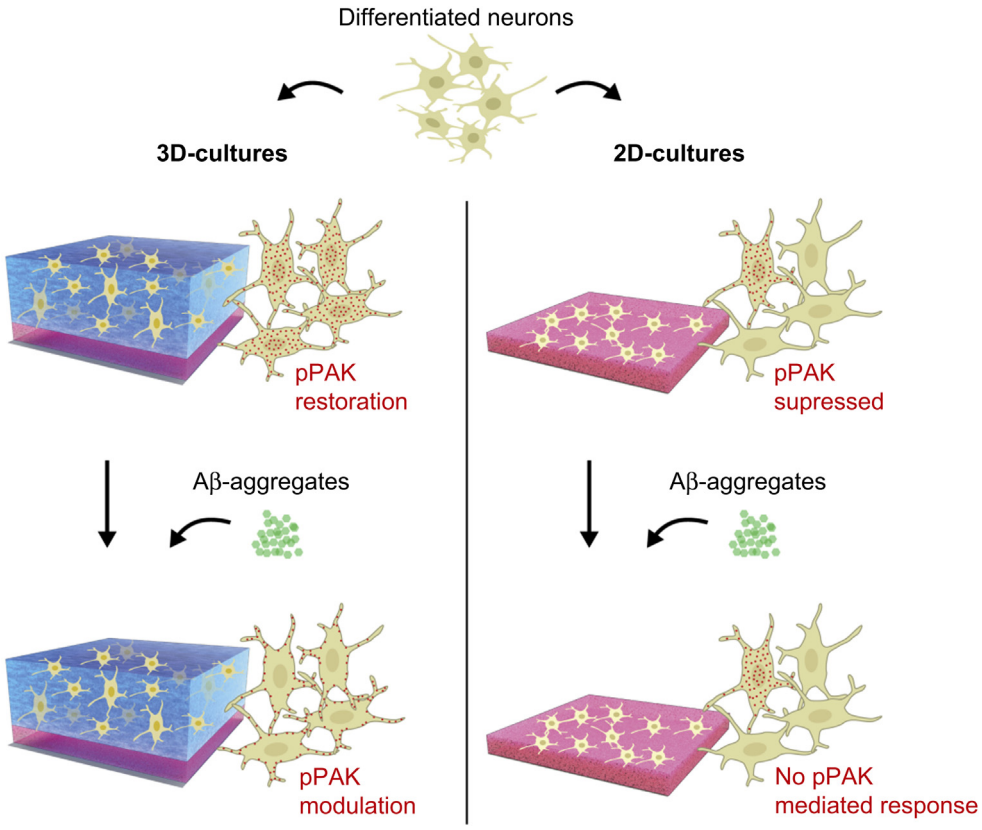

Fig. 9. A sketch illustrating differences in pPAK expression and responses to Aβ oligomers in 2D cultures and 3D SAP matrix cultures.

Alzheimer's disease, drebrin has been reported to be severely lost [22] and cofilin decorated small actin rods have been demonstrated in studies of the disease process [21]. We suggest that in our model the 3D cultured neurons are in a state closer to the *in vivo* situation compared to a conventional 2D culture. The strongest indication of this is that only in the 3D SAP matrix we get a redistribution of pPAK upon stimulation with Aβ oligomers, resembling of what has been observed in other models of AD [20]. A delocalization of drebrin was also seen upon Aβ oligomer stimulation.

5. Conclusions

The conclusions of this study are summarized by a schematic diagram (Fig. 9), which illustrates how a 3D SAP matrix culture of neurons differentiated from NES cells induces high levels of pPAK. Only in this microenvironmental context and not in 2D, Aβ oligomers can induce cellular redistribution of pPAK, which recapitulates pathological changes observed in AD. Moreover this effect is also reflected in other proteins, like drebrin, which are involved in the regulation of actin dynamics.

In our study the 3D neuronal culture, as opposed to a conventional 2D culture, can mimic pathological changes caused by Aβ oligomers, such as redistribution of pPAK and drebrin. Cytoskeleton remodeling is a critical event in the progress of Alzheimer's disease and we propose that the demonstrated 3D culture method can be utilized as a more refined model in studies of mechanisms and possible treatments of the condition.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.biomaterials.2013.11.028.

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