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RESEARCH ARTICLE

Biomaterials directed activation of a cryostable therapeutic secretome in induced pluripotent stem cell derived mesenchymal stromal cells

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Abstract

Mesenchymal stem cell therapy has suffered from wide variability in clinical efficacy, largely due to heterogeneous starting cell populations and large-scale cell death during and after implantation. Optimizing the manufacturing process has led to reproducible cell populations that can be cryopreserved for clinical applications. Nevertheless, ensuring a reproducible cell state that persists after cryopreservation remains a significant challenge, and is necessary to ensure reproducible clinical outcomes. Here we demonstrate how matrix-conjugated hydrogel cell culture materials can normalize a population of induced pluripotent stem cell derived mesenchymal stem cells (iPSC-MSCs) to display a defined secretory profile that promotes enhanced neovascularization in vitro and in vivo. Using a protein-conjugated biomaterials screen we identified two conditions—1 kPa collagen and 10 kPa fibronectin coated polyacrylamide gels—that promote reproducible secretion of pro-angiogenic and immunomodulatory cytokines from iPSC-MSCs that enhance tubulogenesis of endothelial cells in Geltrex and neovascularization in chick chorioallantoic membranes. Using defined culture substrates alone, we demonstrate maintenance of secretory activity after cryopreservation for the first time. This advance provides a simple and scalable approach for cell engineering and subsequent manufacturing, toward normalizing and priming a desired cell activity for clinical regenerative medicine.

KEYWORDS
cryopreservation, cytokines, iPSC-MSCs, stem cell therapy, tubulogenesis

1 | INTRODUCTION

The use of autologous and allogeneic stem cells for tissue regeneration has been at the forefront of regenerative medicine efforts for several decades, with mesenchymal stromal cells (also known as mesenchymal stem cells (MSCs) isolated from bone marrow, adipose tissue, and multiple other sources taking center stage (Pittenger et al., 2019). Although similar, it has to be noted that MSCs derived from different sources differ in terms of surface markers expression, proliferation and differentiation potential, clonality and paracrine activities (Markov et al., 2021). This approach has demonstrated positive outcomes for ischemic heart disease treatment, with low risk of rejection, and revascularization through temporal secretion of trophic, immunomodulatory, and pro-angiogenic molecules (Wu et al., 2019). However, current treatments used for repairing damaged tissue, that are based on the application of stem cells, in particular MSCs, have struggled with respect to reproducible cell delivery and therapeutic efficacy (Ayyat et al., 2019). Recent findings indicate that the most likely mechanism for the beneficial effects of MSC therapy in tissue regeneration is related to the production of bioactive trophic factors that stimulate neighboring parenchymal cells to initiate repair mechanisms in damaged tissues (Fu et al., 2017). In fact, there is no clear evidence that MSC engraftment itself is always beneficial. It has become apparent that the dominant role of MSCs in tissue repair is owed to the release of paracrine immunomodulatory and trophic molecules, and not in the ability of these cells to differentiate into a desired lineage (Murphy et al., 2013; Watt et al., 2013). This conclusion has been derived from multiple studies, where DNA from donor MSCs was not found 12 months post cell implantation, thus supporting the hypothesis that MSC induces a host response to form new tissue, as opposed to a specific differentiation outcome (Caplan, 2017; Huang et al., 2010; Pittenger et al., 2019).

Several studies have indicated that MSC therapeutic potential is tightly linked to the microenvironment in which they are used (Caplan, 2017; DiMarino et al., 2013; Pittenger et al., 2019). A recent paper from Vagnozzi et al. demonstrated that cell therapy enhances heart function but it is not associated with the production of new cardiomyocytes (Vagnozzi et al., 2020). In this respect, matrix stiffness has been reported to influence the behavior of a variety of cell types (Choi et al., 2012; Engler et al., 2006; Forte et al., 2012; Romanazzo et al., 2012; Vining & Mooney, 2017). We previously demonstrated that MSC behavior is affected by the coordinated influence of substrate rigidity and matrix protein composition by using polyacrylamide gels formed through radical addition polymerization (Abdeen et al., 2014). Moreover, other physical cues such as stretch, surface topography and tissue geometry, have been found to affect lineage commitment of stem cells (Bandaru et al., 2020; Kilian et al., 2010; Kshitiz et al., 2012). Mechanical loading, as an example, was shown to influence pro-angiogenic effects of MSCs by increasing their proliferation rate and increasing expression of angiogenesis regulators MMP-2, vascular endothelial growth factor (VEGF), TGF-β1, and bFGF (Kasper et al., 2007).

While there is much promise in the use of stem cells for therapy, there are several hurdles to the clinical viability including time required for isolation and expansion, variability across isolations, and often an immediate need for large numbers of cells. In fact, it is possible to isolate 2×10^3 MSCs from a bone marrow donation, while a cell therapy treatment for a human patient can require more than 1 billion MSCs (Lukomska et al., 2019). An alternative approach involves the use of cells that have been isolated from other donors and banked to be used off the shelf. Allogeneic MSCs are immune privileged and thus can be used with low risk for different patients than the donor (Ankrum et al., 2014). However, MSCs are generally subjected to cell senescence and can be expanded in vitro only for few passages on tissue culture plastic (TCP) (Squillaro et al., 2016). Alternatively, induced pluripotent stem cells (iPSCs), together with embryonic stem cells are pluripotent cells and as such possess indefinite growth abilities and of differentiation into any adult cell type. Although this property is very attractive for in vitro expansion purposes, pluripotent stem cells are not suitable for human administration in their undifferentiated state, due to their propensity to form teratomas in vivo. However, another advantage of pluripotent stem cells is that they have the ability to differentiate into any adult cell type. Consequently, pluripotent stem cells can serve as a starting material for the production of other cell types. Cymerus™ MSCs, originally derived from iPSCs, were directed to differentiate into an intermediate cell type known as mesenchymoangioblasts, that when in semisolid suspension culture form compact spheroid colonies containing mesenchymal cells (Vodyanik et al., 2010). Watt et al. compared different sources of MSC proposing that source, species derivation and identity (primary or cultured cells), can severely affect their performance in vivo (Watt et al., 2013). Because of their generation from iPSC, the Cymerus™ MSC, hereafter called induced pluripotent stem cell derived mesenchymal stem cells (iPSC-MSCs) are regarded as a promising cell source compared to the standard bone marrow derived MSCs (BM-MSCs). These cells have been used in a clinical trial for graft versus host disease with very positive results, and further trials have been approved in critical limb ischemia, osteoarthritis and respiratory distress associated with COVID-19 (Bloor et al., 2018; Khan et al., 2019; Ozay et al., 2019). Taken together iPSC-MSCs show great promise for their trophic, immunomodulatory and pro-angiogenic activity. Nevertheless, it is still believed that differing microenvironments will influence their propensity to secrete bioactive cytokines in a clinically efficacious manner.

In the present study we investigate the culture of iPSC-MSCs on hydrogels of variable stiffness conjugated with a range of extracellular matrix (ECM) proteins to discover optimal matrices to promote cytokine secretion, and to assess the potential of these primed cells in vitro and in vivo. We evaluate the top matrices identified in our screen for promoting neovascularization and identify conditions where the primed state can be maintained after cryopreservation toward cell engineering and manufacture for regenerative therapy.

2 | RESULTS AND DISCUSSION

2.1 | Substrate physical and biochemical composition influences iPSC-MSCs morphology

Induced pluripotent stem cell derived mesenchymal stem cells were first analyzed for their ability to express markers associated with BM-MSCs, such as endoglin (also known as CD105) (Dominici et al., 2006; Lin et al., 2013), with clear evidence for uniform expression (Figure S1). In order to understand how iPSC-MSCs behave on substrates with different stiffness and ECM composition, we cultured cells on polyacrylamide gels with tunable stiffness containing matrix protein covalently bonded at the interface through hydrazone linkages (Abdeen et al., 2014). The range of substrate stiffness was chosen based on ECM mechanics observed in healthy and diseased tissues, reported both in in vitro and in vivo experiments (Discher et al., 2005). Normal myocardial tissue stiffness resides between 5 and 20 kPa. Following myocardial infarction (MI), tissue stiffness raises to 40 kPa up to 90 kPa, because of fibrotic tissue formation (Hiesinger et al., 2012; Shen et al., 2018). Interestingly, it has been reported that 24 h post MI, tissue stiffness decreases to about 4 kPa, to then become significantly higher compared to healthy tissues, from 15 kPa after few days up to 90 kPa 28 days post MI (Zhang et al., 2011). Arterial stiffness has also proven to be an important factor to consider for predicting cardiovascular events (Bonarjee, 2018; Shen et al., 2018). In order to mimic all these conditions in vitro, we developed four types of polyacrylamide gels, having either 1, 10, 40 or 100 kPa. Furthermore, to determine the effect of ECM biochemical composition, we conjugated either collagen, fibronectin or laminin on each substrate. Collagen is one of the most predominant proteins in any ECM of human tissue, constituting up to 30% of its total protein mass (Frantz et al., 2010). Fibronectin is a fibrous protein widely present in the interstitial ECM, with the important role of mediating cell attachment and function (Frantz et al., 2010). Fibronectin has also been found to be implicated in cardiovascular disease, as it is responsible for the correct myocardial precursor migration during heart development (Rozario & DeSimone, 2010). Another important ECM protein in human cardiac tissue is laminin, which is present in the basal membrane of myocytes, smooth muscle and endothelial cells (Yap et al., 2019). To ensure uniform coating of the ECM proteins we employed a bioconjugation approach previously developed (Abdeen et al., 2014), where each protein was oxidized to present aldehydes for covalent conjugation to the hydrazine modified hydrogel.

With the aim of combining mechanical and biochemical factors to influence iPSC-MSCs behavior, we cultured the cells on each substrate for up to 5 days. As expected, when cells adhered to the softest substrates, they acquired a round shape in all three ECM conditions, as shown in Figure 1a, **left** and confirmed by aspect ratio quantification (Figure 1b). 1 kPa fibronectin coated surfaces showed significantly higher cell aggregation compared to the other two ECM conditions (Figure 1a, **middle-left**). On the intermediate 10 kPa gels, cells showed a more elongated shape (Figure 1b), and 10 kPa fibronectin appeared to induce cell alignment compared to the other protein conjugated substrates (Figure 1a, **middle**; Figure S2). Mean cell area quantification showed significant increase in both collagen and laminin coated 10 kPa surfaces, but not in the fibronectin group (Figure 1c). On the stiffest gel coated with fibronectin, cells maintained similar cell spreading, however, they lost cell alignment (Figure 1a, **middle-right**), while 40 kPa collagen substrates showed cell aggregate formation (Figure 1a, **top-right**). All cells cultured on the stiffest substrates presented comparable mean cell area (Figure 1c). Cells cultured on 40 kPa laminin substrates did not change their morphology (Figure 1a, **bottom-right**). Cells cultured on 100 kPa gels showed a spread morphology across all protein coatings (Figure S4).

While collagen and laminin coated gels presented equally spread cells, fibronectin polyacrylamide gels displayed cellular aggregation. Fibronectin is highly expressed in vivo after MI, to attract stem cell homing to the damaged site and subsequent proliferation and differentiation (Frangogiannis, 2017). It was previously shown that fibronectin improves cell attachment and proliferation compared to laminin and collagen, in adipose derived stem cells (Van Dijk et al., 2008), with augmented proangiogenic secretion from BM-MSCs on fibronectin matrices (Abdeen et al., 2014). Thus, we speculate that culture of iPSC-MSCs on fibronectin matrices may foster culture conditions with aspects of native tissue environments, which may prove useful for optimizing a specific bioactivity that may be therapeutically relevant.

2.2 | Combinations of substrate stiffness and matrix protein directs pro-angiogenic activity

To assess the effect of secreted factors released from iPSC-MSCs cultured on different substrates, conditioned media from each group was collected and used to perform an in vitro tube formation assay, where human dermal microvascular endothelial cells (HMVEC) seeded on Geltrex were incubated up to 20 h with media obtained from each group. Representative images of tubes formed are shown in Figure 2a, with correlated quantification (Figure 2b). When comparing iPSC-MSCs conditioned media collected from hydrogel substrates to negative control, where HMVEC were incubated with endothelial cell growth medium without supplements, tube formation was higher in all conditions. HMVEC cultured with media obtained from 10 kPa collagen and 10 and 40 kPa fibronectin (Figure 2a,b), showed the highest degree of tubulogenesis. These groups are also the ones with higher cell aspect ratio (Figure 1d), suggesting that tube formation performance correlates with cell morphological changes on different substrates. Similarly to previous work with BM-MSCs (Abdeen et al., 2014), the iPSC-MSCs preferred fibronectin coated substrates to secrete angiogenic factors; however, they showed higher tubulogenesis on 10 kPa whereas BM-MSCs showed optimal tubulogenesis on 40 kPa fibronectin surfaces. These differences may be on account of cell source and differences in morphology and phenotype (BM-MSCs ~ 0.1 mm^2; iPSC-

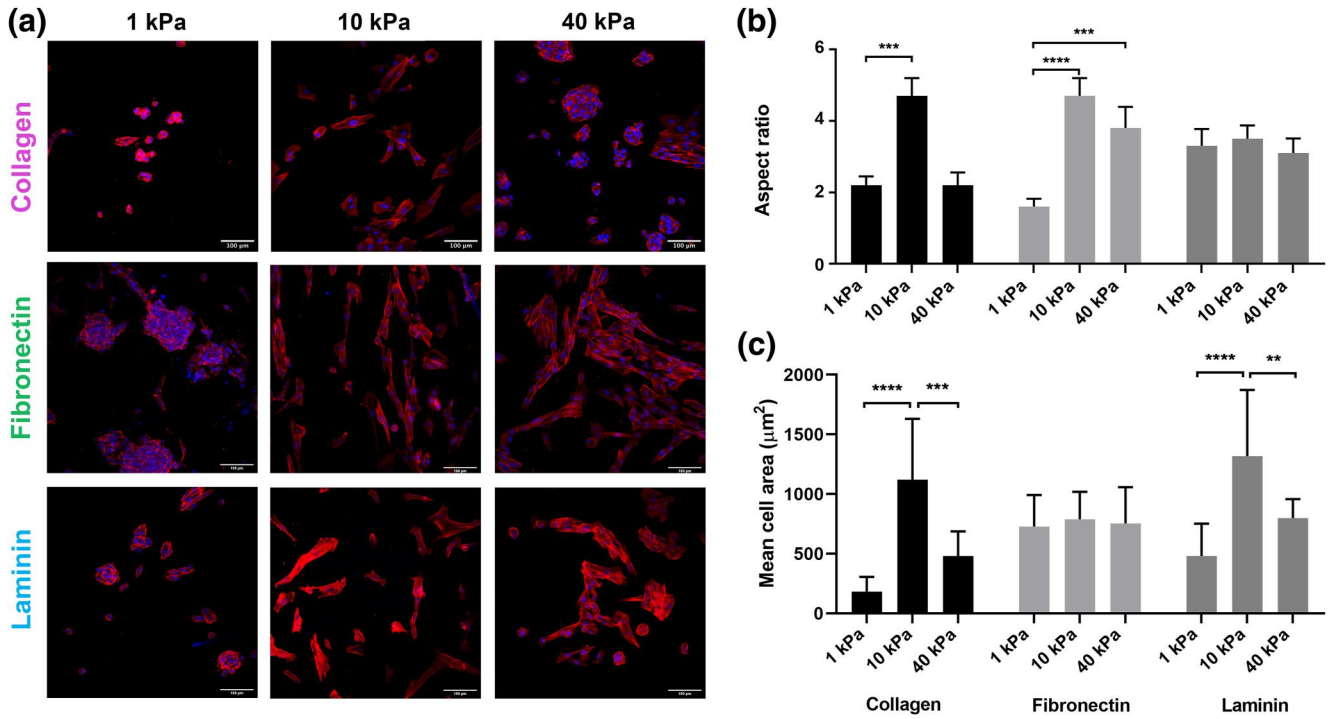

FIGURE 1 iPSC-MSCs show different adhesion characteristics as a function of protein and stiffness. (a) Quantification of induced pluripotent stem cell derived mesenchymal stem cells (iPSC-MSCs) morphometrics when adherent to different hydrogel materials (scale bars: 100 μm), demonstrating pronounced differences in aspect ratio (b) and cell area (c) Results are provided as mean ± standard deviation of $n \geq 3$ independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$

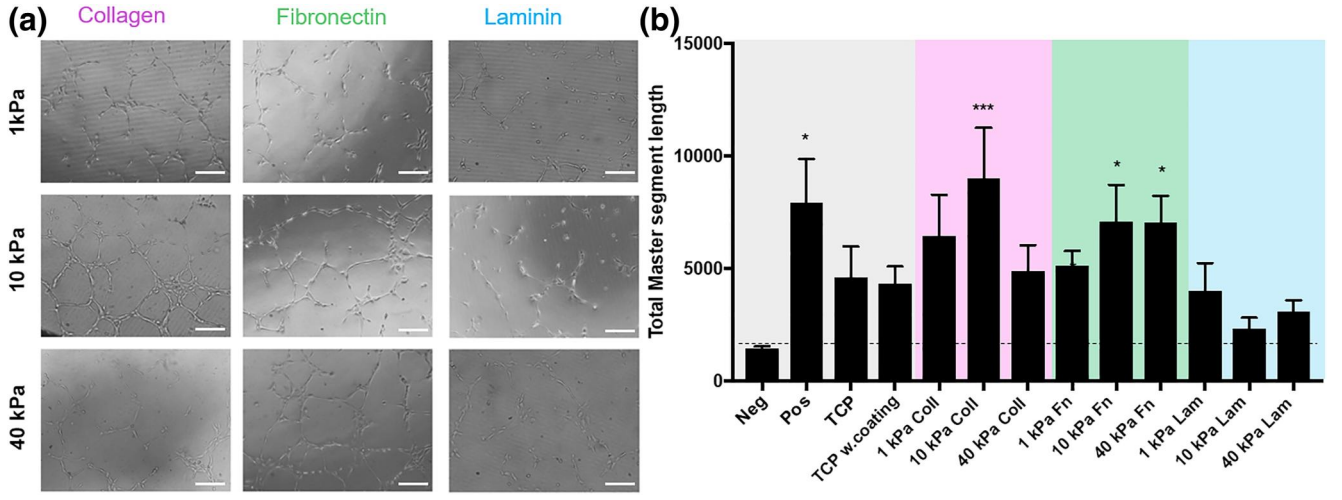

FIGURE 2 Specific culture substrates promote conditions that foster endothelial cell tubulogenesis. (a) Conditioned media from induced pluripotent stem cell derived mesenchymal stem cells (iPSC-MSCs) cultured on polyacrylamide hydrogels of 1, 10 and 40 kPa, conjugated with collagen, fibronectin or laminin, elicits varying degrees of tubulogenesis in human microvascular endothelial cells in 3D Geltrex. Scale bars: 100 μm. (b) Quantification of master tube segment length across all conditions. Neg: unsupplemented EBM-2 media; Pos: fully supplemented EGM-2 media; tissue culture plastic (TCP): media collected from iPSC-MSCs cultured in TCP; TCP w/coating: media collected from iPSC-MSCs cultured in standard culture plastic conditions with coating, as described in Section 4.1; Coll: Collagen; Fn: Fibronectin; Lam: laminin. $^{*}p < 0.05$, $^{***}p < 0.001$

MSCs ~ 0.002 mm^2, Figure S3). Conditioned media from the 10 kPa collagen group showed significantly higher tube formation compared to the positive control with high concentration of supplemented growth factors (Figure 2b, Figure S3). The higher stiffness gels (100 kPa) conjugated with three types of ECM were tested for their ability to influence tube formation; however, they did not show any significant increase compared to the standard approach of culturing cells on TCP (Figure S3B). Conditioned media from iPSC-MSCs on laminin did not show a significant increase compared to media from cells cultured on standard TCP, in any gradient of stiffness tested.

2.3 | Primed iPSC-MSCs maintain their ability to induce tube formation after cryopreservation

Having identified several hydrogels that maximize secretion of factors that promote tubulogenesis, we next sought to evaluate whether this activated cell state would persist. A hallmark of cell manufacturing and therapy is the ability of a cell population to maintain its phenotype during culture and after cryopreservation. Cryopreservation of MSC has proved a considerable challenge, since cell viability, proliferation and differentiation potential are affected by the process of freezing and subsequent thawing (Liu et al., 2011). To evaluate if the primed activity may be maintained during and after storage, we initially cultured MSCs on different substrates for 2 days and performed the first tubulogenesis assay (Figure 3a). At the same time, cells were trypsinized, collected and cryopreserved up to 31 days. Cells were then thawed and seeded in standard TCP for 24 h, and then conditioned media collected from each group was used for assessing tube formation of HMVEC seeded on Geltrex coated dishes (Figure 3b). Before and after cryopreservation, each group maintained the same trend of tube formation ability; however, there were marked differences between culture conditions where the priming persisted to a greater extent: 1 kPa collagen and 10 kPa fibronectin maintained higher number of master segments even after the freeze/thaw cycle (Figure 3b). For this reason, we then moved to the preparation of polyacrylamide gels with either 1 kPa or 10 kPa stiffness values, with a mixed solution containing both collagen and fibronectin conjugated to the hydrogels. Both 1 and 10 kPa mixed groups showed enhanced tubulogenesis compared to TCP. After cryopreservation there was a slight increase in tubulogenesis. However, while this correlates with an increase in pericyte markers, this difference is not statistically significant and requires further investigation (Figure S7).

Cryopreservation of MSCs provides a great opportunity for translating basic research to clinical applications of these cells for a wide range of diseases and disorders, because it: (i) allows the possibility to achieve a steady supply of viable and functional cells, (ii) reduces the constant need for fresh tissues to isolate cells and reduces the batch-to-batch variability enabling quality control and standardization of same cell preparation and banking of cells, (iii) facilitates transportation of cells, (iv) gives time for screening cells for pathogens and transmissible diseases in case of allogeneic applications (Marquez-Curtis et al., 2015). Several groups have investigated the effect of cryopreservation on MSC proliferation and differentiation ability (Bissoyi et al., 2016; Miyagi-Shiohira et al., 2015; Nam et al., 2014; Xu et al., 2021); however, there are no previous reports of cryopreserving MSCs that have been homogenized after culture on defined cell culture materials. Naqvi et al. showed that porcine BM-MSCs in alginate microcapsules primed with TGF-β3 for a period of 2 weeks and subsequently cryopreserved in liquid nitrogen for 72 h, were able to maintain their ability to produce collagen and proteoglycans when thawed (Naqvi et al., 2018). However, this system requires the use of exogenous material and growth factors which may limit its use in a clinical setting. Our approach using optimized cell culture conditions alone, provides a route to cell manufacturing where the combinatorial identification of biophysical and biochemical parameters that prime a specific secretory state may be selected to cryopreserve a desired cellular activity.

2.4 | Substrate stiffness and composition influence cytokine expression

To understand how tubulogenesis is guided by the iPSC-MSCs' conditioned media, we next investigated the expression of 20 cytokines involved in angiogenesis and immunomodulatory activity. The abbreviations for cytokines analyzed are listed in Table S1. Conditioned media was collected from the iPSC-MSCs' cultures after 2 days and applied to cytokine arrays. All groups including 1 kPa collagen, 10 kPa fibronectin and standard TCP, showed considerable elevation of GRO, IL-8 and Timp2 expression compared to the other cytokines (Figure 3c,d). GRO and IL-8 are known to recruit angioblasts in MI areas (Kocher et al., 2006), supporting a role for these molecules in future therapies. The metalloproteinases, such as Timp1 and Timp2 are commonly considered as vasculogenesis inhibitors. However, it has been reported that cells cultured on TCP are stimulated by TIMP-2 (Sang, 1998), which aligns with our results showing that Timp-2 was downregulated in 1 kPa collagen and had lower expression in 10 KPa fibronectin, when compared to TCP (Figure 3d). A number of cytokines known to be involved in angiogenic activity were upregulated both in 1 kPa collagen and 10 kPa fibronectin when compared to TCP, such as EGF and platelet growth factor 2 b subunits (2-fold), ENA-78 (~1.5–2 fold), as well as modest increases in placental growth factor, Rantes, VEGF, b-FGF and angiogenin (Figure 3d). ENA-78, also known as CXCL5, is an important angiogenic factor involved in the inflammatory response during coronary artery syndrome (Zineh et al., 2008). VEGF is historically one of the main growth factors implicated in pathological vasculogenesis triggered by inflammatory response and physiological ischemia (Ridiandries et al., 2016). Rantes, also known as CCL5, is involved in macrophage and monocyte migration to the injured site to promote vasculogenesis. It was previously shown that MSC derived from different tissue sources express Rantes differently, and that adipose derived MSC had significant effects on new vessel formation in ischemic tissue following vascular occlusion (Kimura et al., 2014). The pro-angiogenic effect of iPSC-MSCs was investigated also through the evaluation of either the MSC marker PDGFRβ or the pericyte/smooth muscle marker alpha-smooth muscle actin (α-SMA). Figure S8 shows profiles of the populations cultured on TCP, 1 kPa collagen and 10 kPa fibronectin before and after cryopreservation. While we observed a decreased expression of the stemness marker PDGFRβ, there was no alteration of α-SMA expression after cryopreservation for cells cultured under normal conditions (TCP). Both hydrogels conditions also showed a slight decrease in the expression of PDGFRβ, though they seem to maintain a more stable expression after cryopreservation. Interestingly the hydrogel groups had higher fraction of cells expressing α-SMA compared to

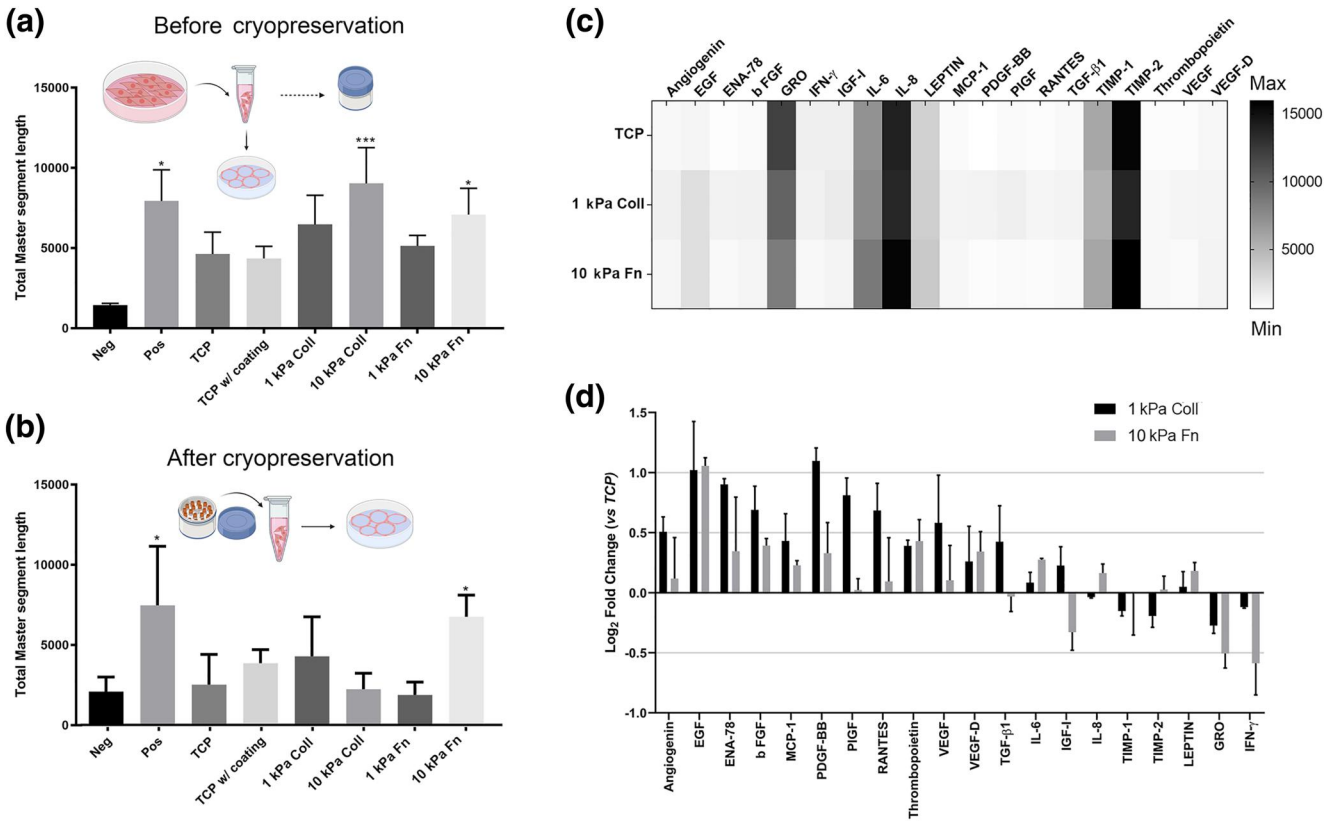

FIGURE 3 iPSC-MSCs activity remains after cryopreservation. (a) Conditioned media from mesenchymal stem cells (MSCs) enhance varying degrees of tubulogenesis in Geltrex encapsulated endothelial cells. (b) After cryopreservation the induced pluripotent stem cell derived mesenchymal stem cells (iPSC-MSCs) maintain the pro-angiogenic potential under several conditions. $^{*}p < 0.05$, $^{***}p < 0.001$; (c) Cytokine array profiling demonstrates how the two most pro-angiogenic coatings (1 kPa collagen (Coll) and 10 kPa fibronectin (Fn) stimulate increased secretion of a broad range of factors involved in angiogenesis compared to tissue culture plastic (TCP). (d) Quantification of cytokine array experiments showing fold change in secretion over the TCP control

TCP post-cryopreservation, suggesting that iPSC-MSCs culture on the selected substrates promotes the release of a pro-angiogenic secretome. The precise mechanism through which this phenomenon takes place remains unclear. PDGFRβ has been identified as marker for MSCs that present enhanced pro-angiogenic property within an heterogenous MSC population (Wang et al., 2018). In fact, this receptor is also considered to be highly expressed in pericytes (Andrae et al., 2008). Thus, the higher expression of PDGFRβ and increased detection of α-SMA in the hydrogels groups confirms the positive pro-angiogenic effect of these substrates.

2.5 | Integrin mediated adhesion promotes pro-angiogenic activity

The top hydrogels that facilitate enhanced secretion of pro-angiogenic factors show differences in cell morphology. The ability for a cell to adapt to the ECM first involves adhesion using integrin surface receptors, followed by dynamic traction of the matrix to probe mechanical properties through actomyosin contractility. To assess whether actomyosin contractility on the substrates is linked to the pro-angiogenic secretome, we treated our cultures with the non-muscle myosin inhibitor blebbistatin at concentrations that facilitate adhesion (1 μM) and do not adversely affect cell morphology (Figure 4a). At 48 h the conditioned media was applied to hMVECs on Geltrex to study how actomyosin contractility inhibition may inhibit secretion of pro-angiogenic molecules through the functional tubulogenesis assay. In each tested condition, treatment with blebbistatin abolished tube formation (Figure 4b, **top**).

Next, we asked whether perturbing adhesion would influence the pro-angiogenic behavior of the MSCs through the use of a $\alpha_V\beta_3$ blocking antibody, supplemented into the MSC media at 1 μg/ml (Figure 4a). We selected this concentration as it does not perturb gross cell morphology or adhesion to the substrate, while simultaneously reducing integrin-mediated downstream signaling (Kilian et al., 2010). iPSC-MSCs adherent to our hit substrates showed robust $\alpha_V\beta_3$ immunostaining before and after cryopreservation, particularly when adhering to substrates containing fibronectin (Figure S9). Treatment with blocking antibodies for $\alpha_V\beta_3$ abolishes tubulogenesis in hMVECs exposed to conditioned media from cells cultured on matrices containing fibronectin, with some partial attenuation of tubulogenesis in hMVECs exposed to conditioned media from cells cultured on collagen I (Figure 4b, **bottom**). This is consistent with the role for $\alpha_V\beta_3$ integrins as major receptors for

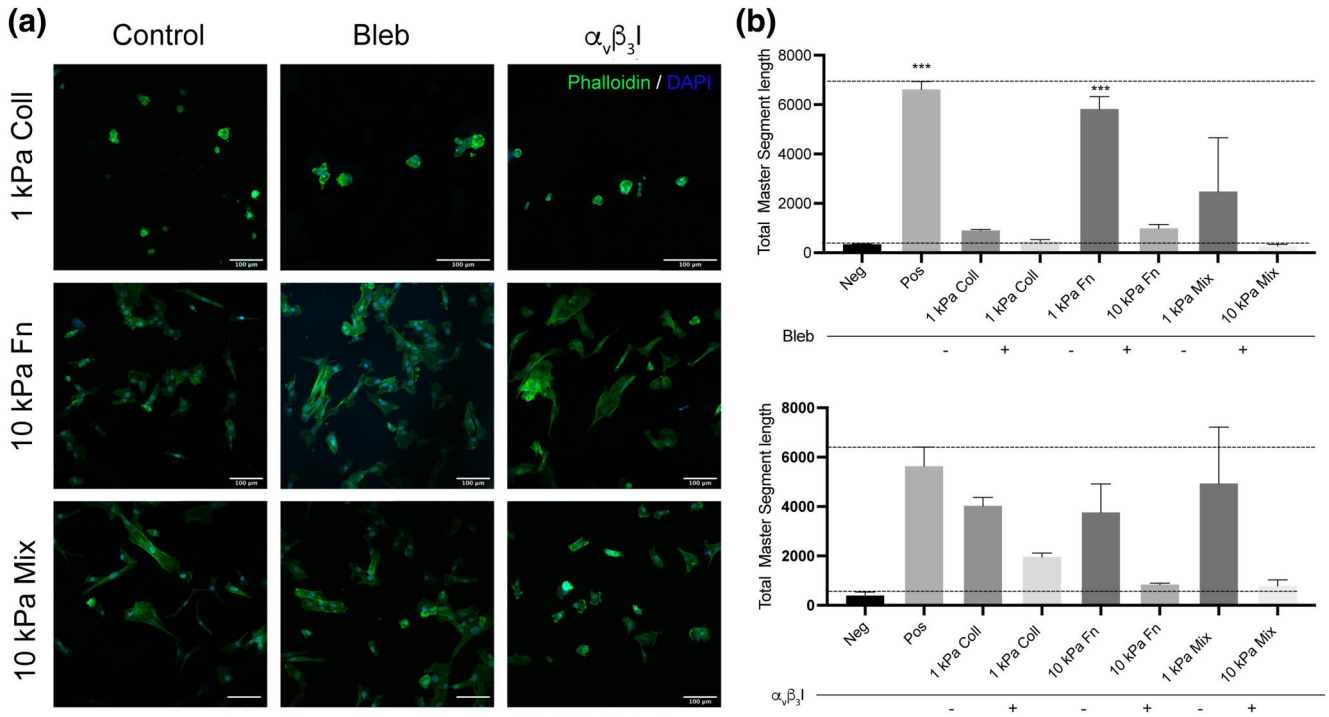

FIGURE 4 Integrin adhesion and actomyosin contractility regulate the secretome from mesenchymal stem cells (MSCs). (a) Immunofluorescence images of induced pluripotent stem cell derived mesenchymal stem cells (iPSC-MSCs) adherent to 1 kPa Coll, 10 kPa Fn and the 10 kPa mixed condition (1:1 Coll:Fn) with and without blebbistatin treatment (Bleb) or integrin blocking ($\alpha_V\beta_3$I). (b) Quantification of the degree of tubulogenesis in hMVECs on Geltrex after exposure to conditioned media from MSCs treated with Bleb or $\alpha_V\beta_3$I. Coll: Collagen, Fn: Fibronectin, Mix: Collagen and Fibronectin. Scale bars: 100 μm

adhesion to fibronectin, and suggests that the pro-angiogenic secretome is triggered through actomyosin contractility generated by integrin $\alpha_V\beta_3$ adhesion.

2.6 | Primed iPSC-MSCs maintained angiogenic properties in vivo

To confirm our in vitro findings, we used a chick chorioallantoic membrane (CAM) in vivo model of angiogenesis, where we loaded conditioned media collected from each hydrogel cell culture. Chorioallantoic membrane assays are a common in vivo angiogenesis assay in which molecules, cells or materials of interest are placed on the vascularized CAM which surrounds a fertilized chicken embryo and changes in the CAM vessel density are observed. Vessel formation was analyzed for samples collected before and after the freeze-thaw cycle, in conjunction with our in vitro study. As 1 kPa collagen and 10 kPa fibronectin showed the highest tube formation compared to other groups, we decided to move forward with these two hydrogel formulations, and to add a mixture of collagen and fibronectin in 1:1 ratio, to determine if a combination of our optimal ECM conditions would further enhance neovascularization. Conditioned media collected from cells before and after cryopreservation showed a significantly higher ability to induce branch formation in the CAM assay compared to full media only and phosphate-buffered saline (PBS) (Figure S7). The CAM assay also confirmed that our matrices are able to induce more vessel formation than cells cultured on TCP. Moreover, when collagen and fibronectin were combined on the 10 kPa hydrogel, the combined effect resulted in a significantly higher tube formation yield compared to TCP and to single coated substrates too (Figure 5a,b). Strikingly, when we thawed the iPSC-MSCs that had been cryopreserved for 1 month and seeded them on TCP for 24 h, conditioned media from these cells were able to form vessels following the same trend observed before cryopreservation (Figure 5c,d).

3 | CONCLUSION

Cell-based therapies have fallen on hard times, where clinical efficacy has suffered from inconsistences in isolation, reproducibility, and lack of insight into mechanisms of action. Here we explored the use of the Cymerus™ technology from Cynata Therapeutics, which are single donor iPSC derived MSCs, and identified precise cell culture conditions that maximize the secretory and immunomodulatory profile from the iPSC-MSCs. We showed that iPSC-MSCs behave differently depending on matrix stiffness and matrix composition, in terms of adhesion and proliferation characteristics, and the ability for the conditioned media to induce neovascularization. After screening a library of protein conjugated hydrogels with various media formulations, we identified conditions where the secretory state of the cells could be preserved after cryopreservation, thus paving the way to

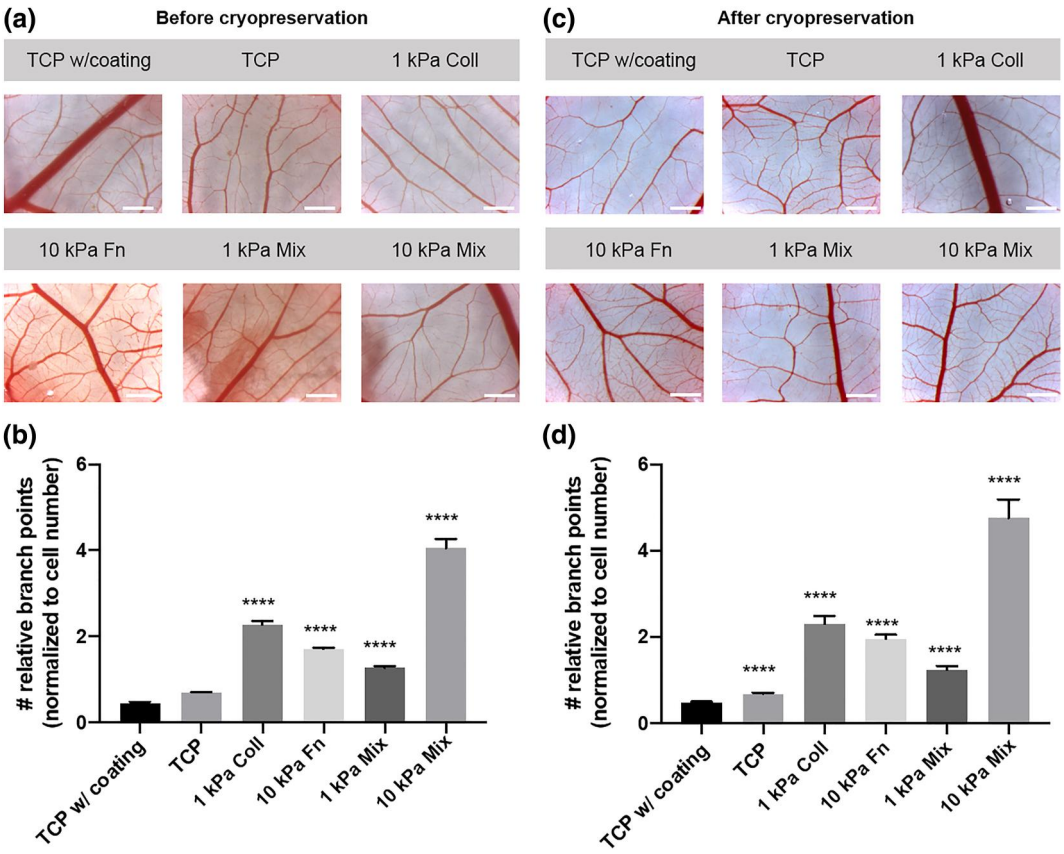

FIGURE 5 Primed induced pluripotent stem cell derived mesenchymal stem cells (iPSC-MSCs) facilitate neovascularization in chick chorioallantoic membranes. (a) and (c) show representative images of chorioallantoic membrane (CAM) of each experimental group before and after cryopreservation respectively. Scale bars: 1 mm. (b) and (d) represent quantification of number of branches formed, normalized to number of cells from which each media group was collected and used for CAM assay. $^{****}p < 0.0001$. TCP w/o coating: Tissue culture plate without coating; TCP: tissue culture plastic (TCP) pre-coated with fibronectin/collagen solution described in Section 4.1; Coll: collagen type I; Fn: fibronectin; Mix: solution containing 50% collagen type I and 50% fibronectin

manufacturing approaches where a desired cell state may be maximized and normalized across a population. Here we explored the secretion of a panel of cytokines related to angiogenesis and immune modulation; the same approach could be leveraged to identify culture conditions that maximize virtually any desired activity to suit the application. Integrating allogeneic cells with advanced cell culture substrates has the potential to broaden the suite of cell-based tools for regenerative medicine, thereby overcoming many of the hurdles that have precluded broad scale use in clinical settings.

4 | EXPERIMENTAL SECTION/METHODS

4.1 | Detailed methods can be found in supporting information

4.1.1 | Cell culture

Induced pluripotent stem cell derived mesenchymal stem cells, provided by Cynata Therapeutics, were cultured on TCP pre-coated with Fibronectin/Collagen solution per manufacturer instructions. Briefly, iPSC-MSCs (passage 4–9) were seeded at 1.3×10^4 cells/cm^2 density and passaged at 70% confluence using Accutase and seeded at 5000 cells/cm^2. After 2 days of culture, conditioned media from each group was collected and used to perform tubulogenesis assay. Adult human dermal microvascular endothelial cells (HMVEC; Lonza) were cultured in fully supplemented EGM-2 media. Inhibitors were added to cells as described in supporting information materials and methods.

4.1.2 | Gel fabrication and microprinting

Polyacrylamide gels were made as described previously (Abdeen et al., 2014). Briefly, 18-mm coverslips were activated by treatment with 0.5% 3-aminopropyltrimethoxysilane (APTS) followed by 0.5% glutaraldehyde solution. 40% acrylamide and 2% bis-acrylamide monomers with 10% ammonium persulphate and 0.1% tetraethylmethylenediamine (TEMED) were mixed at ratios to obtain gels with different stiffnesses. Hydrogel samples were treated with hydrazine hydrate followed by 5% glacial acetic acid. Proteins oxidized with sodium periodate were patterned using soft lithography.

4.1.3 | In vitro tubulogenesis assay

The in vitro tubulogenesis assay was performed as previously described (Abdeen et al., 2017). Briefly, 1.5×10^4 HMVECs were seeded in GelTrex coated wells, with each well containing 100 µl of unsupplemented EBM-2 media (Lonza, USA) and 400 µl of conditioned media, collected from each experimental group. Tube formation was assessed up to 24 h using an Olympus CKX53 microscope at 20X magnification and analyzed using ImageJ plugin "Angiogenesis analyzer".

4.1.4 | Immunofluorescence and flow cytometry

For immunofluorescence, cells were fixed with 4% paraformaldehyde solution, permeabilized in 0.1% Triton X-100 and incubated with primary antibody solution in 1% bovine serum albumin for 1 h at room temperature. Secondary antibody solutions were incubated with the cells per manufacturer instructions. Samples were mounted and imaged with the Nikon A1 confocal microscope. For flow cytometry, cells were collected via trypsinization, and fixed and stained using similar procedures. Cells were analyzed using a BD LSR Fortessa flow cytometer and FlowJo™ v10 software.

4.1.5 | Chorioallantoic membrane (CAM) angiogenesis assay

Fertilized Barter black chicken eggs were opened and processed as described previously (Kimura et al., 2014). On day 6, a silicone ring was placed on top of the CAM and 50 µl of sample (conditioned media, PBS or VEGF165) was added per ring. On day 11, the CAM was excised around the silicone ring and imaged under a dissecting microscope at 2.5X magnification. Number of blood vessel branch points in 20 mm^2 area were counted using ImageJ.

4.1.6 | Statistical analysis

Statistical analyses were performed using GraphPad Prism (version 8) software with at least three samples analyzed for each experimental group. Unpaired *t*-test was used to compared 2 groups, whereas one-way or two-way analysis of variance (ANOVA) was used for ANOVA with Bonferroni's post-tests to compare between groups.

4.1.7 | Supporting information

Supporting Information is available from the Wiley Online Library or from the author.

AUTHOR CONTRIBUTIONS

Sara Romanazzo, Chantal Kopecky, Shouyuan Jian, Riddhesh Doshi, Vipul Mukund, and Pallavi Srivastava performed the research. Sara Romanazzo, Kilian Kelly and Kristopher A. Kilian designed the research approach and methods. Jelena Rnjak-Kovacina and Shouyuan Jian contributed essential tools and analysis. Sara Romanazzo, Chantal Kopecky, and Shouyuan Jian analyzed and interpreted the data. All authors contributed to writing the paper.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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