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Unveiling mesenchymal stromal cells' organizing function in regeneration

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Abstract

Regeneration is a fundamental process much attributed to functions of adult stem cells. In last decades delivery of suspended adult stem cells is widely adopted in regenerative medicine as a leading mean of cell therapy. However, adult stem cells can not complete the task of human body regeneration effectively by themselves as far as they need a receptive microenvironment (the niche) to engraft and perform properly. Understanding of mechanisms underlying mammalian regeneration lead us to an assumption that improved outcomes of cell therapy requires a specific microenvironment generated in damaged area prior to stem cell delivery. To certain extent it may be achieved by delivery of mesenchymal stromal cells (MSC), not in dispersed form, but rather self-organized in cell sheets (CS) – tissue-like structures comprising of viable cells and microenvironment components: extracellular matrix and soluble factors deposited in the matrix.

In this communication we highlight a potential role of mesenchymal stromal cells (MSC) as regeneration organizers and speculate that this function emerges in CS. This concept shifts our understanding of therapeutic mechanism underlying a widely known CS-based delivery method for regenerative medicine.

Keywords: stem cell, stromal cell, mesenchymal stromal cell, regeneration, cell sheet, cell delivery

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1. The challenge for stem cell therapy: importance of microenvironment

In last decades stem cell therapy has moved from use of fetal and embryonic material to adult stem cell application pursuing the goal of damaged tissue structural and functional restoration [1]. Adult stem cells localized in majority of organs driving their physiological renewal and recovery after damage or disease [2]. This natural mechanism of human body regeneration provided a promising tool for regenerative medicine and resolved ethical problems related to fetal material use as well as major safety concerns connected with use of undifferentiated and/or potentially teratogenic cells [3, 4].

Despite initial promise of stem cell therapy it showed limited efficacy in numerous clinical trials [5] and, thus, put to doubt initial concept portraying adult stem cells as regenerative "commando engineers" that can engraft, differentiate and rebuild fully functional tissue once delivered viable and in sufficient amounts [6]. Eventually, accumulated data led to a conclusion that most of delivered stem cells fail to integrate and give rise to new tissue elements and work as "bystanders" releasing paracrine factors and disappearing within several weeks [7-9].

Discussing reason of disappointing results of stem cell therapy we may question what can be improved to overcome these hurdles in the way of the promising approaches in biomedicine. To answer that crucial role of microenvironment should be considered – from location of cell to set of signals required to maintain finely tuned physiological functions [10]. This issue is known since Raymond Schofield's seminal work suggesting that stem cells are "non-autonomous" and depend on microenvironment known as *niche* [11]. The latter controls, directs and supports balanced stages of stem cell life cycle and includes other cells (stromal, parenchymal, vascular etc.), extracellular matrix (ECM), soluble proteins and peptides, extracellular vesicles, small molecules and chemical factors (pH, oxygen pressure etc.) [12].

Importance of microenvironment for success of stem cell-driven body repair has been supported by investigation of regeneration in animals with ability to rebuild body parts or even whole organism after injury (e.g. planarians, starfish, axolotl). In

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these creatures "separated" adult stem cells (neoblasts) themselves lack ability to drive full-scale regeneration and require certain amount of tissues to preform properly [13].

Human adult stem cells administered to damage site in suspension are deprived of crucial stimuli required for their regenerative potential to unfold. They lack appropriate intercellular contacts, nutrition and regulatory signals. Under such unfavorable conditions formation of mature tissue elements from transplanted stem cells is an extremely rare event hardly providing significant therapeutic impact [14, 15].

In this paper we summarize our vision on problems that stand in the way of successful application of stem cell therapies. We focus on a cornerstone role of microenvironment formed during regeneration and contribution of mesenchymal stromal cells (MSC) to this process. We suggest that tissue engineered constructs known as cell sheets (CS) present a feasible tool to unfold potential of MSC as organizers of regeneration.

2. Shifting the focus to microenvironment: feeder needed!

To illustrate the possible reason of stem cell therapies failure and support our accent on importance of receptive microenvironment for success of therapeutic approach we shall start with a metaphor to compare mammalian tissue regeneration with repopulation of an ecosystem after a natural catastrophe.

It is well-known that structure of ecological systems has hierarchy based on food chains. It is typically portrayed as a pyramid reflecting dependency of high-order consumers on lower-order and down to the ground level of energy-absorbing producers – plants and microorganisms (left part of Figure 1).

After disaster hierarchy of ecosystem and its structure recovers stage by stage from the foundation starting with the most adaptive and viable species - producers. When they generate a necessary trophic substrate then more demanding species can enter this system and populate it. Gradually the species diversity of entire ecosystem increases and populations begin to interact controlling each other until hierarchy of

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ecosystem is restored and recovery occurs [16]. However, the cornerstone of this process is – and it should be emphasized – formation of a "ground level" of producers required for other species to survive [17].

This metaphor was used to show why therapies using stem cells may fail. In acute phase of response to injury we deliver them to a microenvironment lacking necessary elements and regulatory framework that existed before damage. We speculate that stages of ecosystem recovery might portray regeneration in humans and illustrate that stem cells are capable to unveil their potential only when adequate microenvironment is generated prior to that. Indeed, after damaged area is cleansed by inflammatory cells, they leave "ground zero" with disrupted structure of the tissues down to molecular level. Under such conditions stem cell fails to rebuild the tissue as it depends on other elements – ECM, soluble factors, endothelium, stromal cells and neural terminals disrupted by damage [10-12].

In ecological terms this may be described as a high-order consumer entering the vast field of ashes after a forest fire before the lower levels are re-populated. Failing to find food and mate eventually he will be leaving the unsuitable habitat. Following this idea basal layer of right side of the pyramid - the *feeder* becomes of paramount importance for microenvironment rebuilding to involve specific progenitors (right part of Figure 1) similarly to the ecological dogma that producers presence is obligatory prior to consumers' population appears.

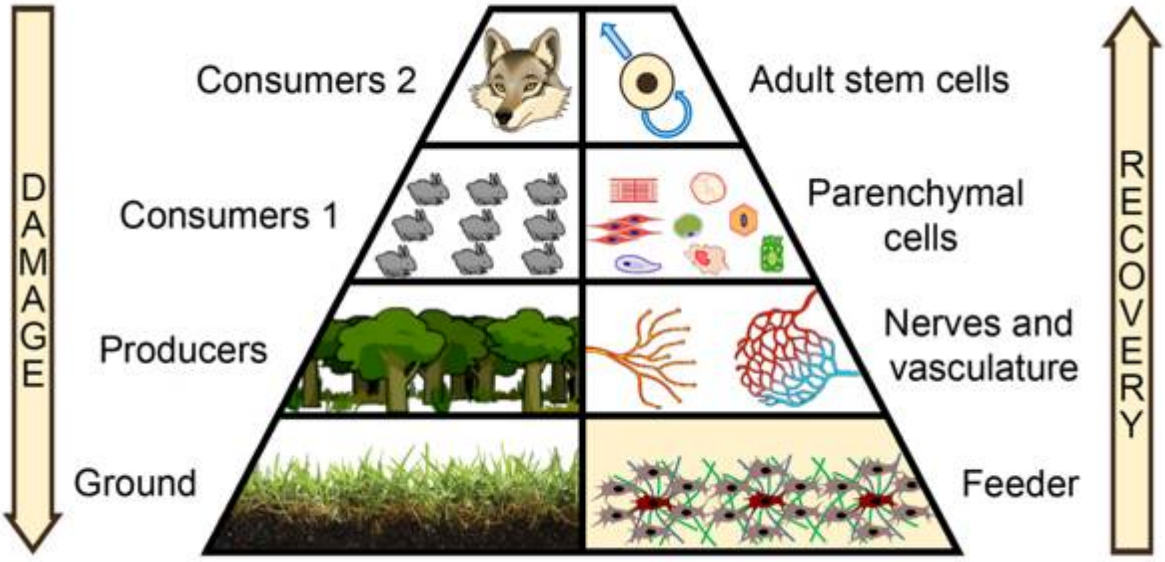

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Figure 1. Critical role of feeder in sequential regeneration of human body.

Both in ecosystem and human body "ground" and "feeder" levels respectively are generated by the most adaptive and universal inhabitants. They have critical importance as they provide foundation for subsequent interactions between elements and the system structure recovery (see text for detail).

We define the *feeder* as a transient heterogenous structure formed *in vivo* at early stage of regeneration to re-create disrupted components of microenvironment which allows other cell types to perform properly in recovery of structure and function. In our concept this putative feeder may contain stromal, immune and probably vascular cells and intercellular components of microenvironment: ECM and soluble factors predominantly anchored in the matrix to render paracrine effects and create gradients of stimuli mapping further stages of regeneration.

Similar requirement for feeder has been described in zoology prior to formation of blastema after amputation of a limb in salamander. Immediately after injury a layer of epidermal cells covers the wound and becomes an *apical epithelial cap* providing ground for attraction of fibroblasts and other cells that dedifferentiate to form the blastema and eventually regrow the limb [18].

An encouraging example of whole organ recapitulation from delivered stem cells in humans is bone marrow transplantation [19]. However, in this procedure after myeloablative stage the stromal component of bone marrow remains relatively intact providing a functional niche for transplanted hematopoietic cells supporting the idea that receptive microenvironment is critical for regeneration allowing stem cells to rebuild tissue but not work as secretome-producing "bystanders" [14, 15].

Typical example of a non-receptive microenvironment that can be formed after damage is scar – avascular bulk of ECM packed to hold the tissues together and barely undergoing resolution over time. Stem cell therapies have shown little to no effect in stimulation of fibrosis resolution yet in some disorders like cystic fibrosis or idiopathic pulmonary fibrosis they have been shown to attenuate disease

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progress [20]. Nevertheless, these effects were once again generally attributed to cells' paracrine function rather than engraftment and tissue restoration [8].

3. Re-building the feeder: focus on mesenchymal stromal cells (MSC)

Thus, we come to a conclusion that stem cell therapies do not achieve their goal until a receptive microenvironment is being formed. Due to a defined order of events during regeneration after injury we suggest to focus not only on tissue-specific stem cells but on the basal layer for which we provide the term *feede*r (in particular, similarly to cell culture technique used to support stem cells and other cell types). Below we speculate on participation of MSC in its formation *in vivo* and address their biological and physiological functions that support this point.

Among numerous cell types of human body MSC remain a prominent candidate for therapeutic use and in our concept they comprise an important (yet not the only) component of putative feeder. In human body MSC are involved in maintaining of structure and homeostasis [21] comprising an ubiquitous stromal component in majority of organs. In quiescent state they are localized in perivascular compartments supporting vascular permeability, secrete growth factors, support ECM production and turnover. These functions are important for interaction with local tissue progenitors and stem cells to retain organs architecture by maintaining gradients of stimuli. In intestinal crypt niche MSC create a gradient of bone morphogenic proteins (BMP) that controls resident stem cell fate and differentiation [22]. However, this tissue function of MSC is not limited to stem cell niches and in intact organs they participate in formation of microenvironment for all cells located nearby [23].

After damage MSC migrate to the lesion site where they actively proliferate [24]. Their ability to survive under stress resulted in excellent cultural properties of isolated MSC. Upon cease of acute phase of inflammation MSC attenuate intensity of residual inflammation. They secrete a wide range of anti-inflammatory factors (IL-10, TSG-6, PGE_2) and, thus, switch the microenvironment to a pro-regenerative state reducing oxidative stress and toxic compound production by immune cells [25].

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Further, MSC produce ECM proteins, and factors of matrix turnover (matrix metalloproteases etc.), as well as cytokines and growth factors to attract and support other cell types (endothelial, tissue-specific progenitors etc.) [25-27].

This guided to investigate the composition of MSC secretome and our group as well as others found that physiological potency of secretome is sufficient to trigger events crucial for regeneration [28-30]. It activates proliferation and survival of different cells under stress, protects tissue from excessive fibrosis, induces angiogenesis and nerve growth, regulates immune response etc. leading to idea of secretome therapeutic application known as "cell-free cell therapy" [31-33]. Our further efforts were focused on functionalization of MSC secretome by viral delivery of growth factors genes or pre-treatment by recombinant proteins. And we have shown that it results in significant shift of MSC functional activity and improvement of therapeutic effect [34-36]. However, we did not find increase of engraftment after functionalization leading us to a generally supported conclusion that MSC therapies succeed to deliver secretome produced by stem cells over time rather than replenish native tissue elements by their differentiation.

Table 1. Biological properties of MSC that contribute to their potential as participants of feeder formation

MSC property/function	Reference
Ubiquitous location in tissues and organs	Bianco, et al. [37], Caplan, et al. [38]
Multipotency and high proliferative potential	Pittenger, et al. [39] Kalinina, et al. [40]
Survival under stress conditions and inflammation	Lee, et al. [41] Oh, et al. [42] Grigoryeva, et al.
Immunomodulation and reduction of inflammation	Bernardo and Fibbe [26] Spaeth, et al. [43] Semedo, et al. [44]

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Active pleiotropic secretome comprising of ECM components, growth factors, cytokines and extracellular vesicles	Kalinina, et al. [28] Konala, et al. [29] Vizoso, et al. [32]
Ability to support and regulate other cell types *in vivo*	Rubina, et al. [27] Battiwalla, et al. [45]

Thus, after injury damage-activated MSC mitigate inflammatory response and produce basic elements of the putative *feeder*: ECM and soluble trophic factors to attract other cells, to interact with them, to form a basis for regeneration.

Nevertheless, one may wonder why MSC delivered by injection fail to form interactions between each other and drive regeneration. Available answer is that negative influence of ECM and contacts disruption due to harvesting from monolayer followed by injection makes MSC unable to perform their functions [46]. It is predominantly related to stress due to loss of microenvironment that existed *in vitro*, namely – adhesive surface, ECM and soluble factors in culture medium. Certain reports claim that after injection up to 70% of MSC undergo cell death within the next 48-72 hours after delivery [40].

Thus, MSC therapy is limited to bystander effects similar to other stem cell therapies. However, MSC are able to produce basic components of their own microenvironment: ECM and pro-survival soluble factors. Moreover, they are able to organize this microenvironment and generate a tissue-like structure *in vitro* – namely, cell sheet (CS). The latter is generally used to facilitate cell delivery and transplant MSC in an organized manner resolving the problem of dispersed cells' inability to survive without appropriate microenvironment. Below we shall provide our point of view and data that support CS-based MSC delivery as a more physiological approach potentially providing ground for effective human regeneration.

4. Cell sheet technology: basics and application

Cell sheets are scaffold-free tissue-like constructs consisting of living cells, ECM and soluble factors accumulated in routine culture conditions. Formation of

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CS varies in time (3-14 days), depends on ECM and soluble factors production, cells proliferation and their ability to integrate to a multilayered construct that detaches from thermo-responsive coating [47] or after treatment by chelating agents and/or mechanical peeling [48].

This technology quickly found application in many areas of regenerative medicine, primarily due to advantages over direct injection. Delivery by CS is positioned as a method to deliver cells along with microenvironment formed *in vitro*. Many preclinical and clinical studies report CS efficacy to treat diseases of the bones, cartilage [49, 50], skin, urinary bladder, heart, blood vessels, esophagus, cornea etc. [51]. To better understand details and therapeutic promise of CS technology in regenerative medicine we may address the Reader to a number of recent seminal reviews [52-56].

Experimental studies to treat wounds, limb ischemia [57], nerve damage [58] and myocardial infarction [59] have been published by our group during last decade. Besides delivery of primary MSC sheets we have studied endothelial cells, cardiac stem cells [48] and viral modification of CS to enhance therapeutic outcomes [57, 58, 60] and our data along with published works of other researchers allows two general conclusions regarding CS as a delivery platform for MSC therapy.

1. Delivery of MSC assembled as CS results in strong and reproducible increase of efficacy in models of different tissue lesions.

Comparison of efficacy after delivery of equivalent amount of MSC by injection or by CS transplantation has shown that sheets are superior to injected cells. In our studies CS have performed better than suspended MSC for treatment of ischemia, nerve repair [57, 58, 61], deep wound healing [62] and pressure ulcer (data unpublished). Recent papers describing use of CS for liver regeneration [63] or bone healing [64] often omit direct “head to head” comparison of outcomes after CS or suspended delivery yet most of them report long-term survival of transplanted cells suggesting that it mediates therapeutic outcome.

2. MSC delivered as CS show signs of engraftment including long-term survival (over 14 days), vascularization of CS and proliferation of delivered cells.

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This point was illustrated in one of our works using subcutaneous implantation of labeled MSC sheets in mice indicating CD31-positive capillaries and larger caliber blood vessels in CS mass as well as Ki-67-positive MSC detected 2 weeks after transplantation [57]. Our results were supported by data from other groups using MSC sheets [65-67] and by own data using other cell types – namely cardiac stem cell sheets transplanted to treat myocardial infarction in a rat model [48, 59].

Points mentioned above support our suggestion that assembly of CS from MSC facilitates *engraftment* and, thus, delivery of a stromal part of suggested feeder that may become a putative basis for further regeneration. However, to do this MSC must interact with other cell types and integrate in an organotypic structure. Potential mechanism for that is generation of stimuli gradients which will be explained further.

5. Role of MSC in structure organization in development and regeneration

Early stages of human development are driven by embryonic stem cells' (ESCs) ability to interconnect and integrate in community [68]. Crosstalk between ESCs dynamically changes form and structure resulting in embryogenesis. Mesenchyme appears during gastrulation phase and gets involved in subsequent organogenesis. Interactions of mesenchymal cells with epithelial cells are crucial in development of teeth [69], lungs [70], pancreas [71], heart [72], kidney [73, 74] and liver [75].

Critical aspect of mentioned processes in development is formation of stimuli gradients including those produced by mesenchymal cells [76]. Soluble factors, ECM and cell-to-cell contacts have been postulated to participate in accurate organogenesis of many organs and role of mesenchymal cells acting as main structure organizers is well-established as well [77, 78].

In postnatal period MSC (despite losing many traits they featured in development) may retain organizing ability they use for regeneration and molecular

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basis of this ability is generation of stimuli gradients once regenerative program is activated by injury. This supports our concept of MSC role in generation of putative feeder and explain how they can facilitate and "map" next stages of regeneration.

6. MSC as organizers of other cell types: *ex vivo* evidence

MSC's ability to support and organize other cells *in vitro* has been reported previously for epithelial cells from embryonic salivary gland. In this study MSC but not conventionally used feeder (NIH/3T3 fibroblasts) supported self-assembly of primary embryonic epithelial cells and subsequent branching with buds formation in 3D culture resembling mesenchyme function in development [79].

One of the strongest evidences of MSC ability to facilitate organogenesis *in vitro* is a work by Takebe et al. [80] describing MSC-driven condensation of iPS-derived progenitors to form bud-shaped organoids. Spectrum of organoids that can be successfully assembled under influence of MSC *in vitro* included liver, intestine, lung, kidney, heart and brain (!). Finally, endothelium-containing pancreatic and renal buds derived under influence of MSC were transplanted to animals where they rapidly underwent vascularization and self-organized into functional, tissue-specific structures.

Organization of such complex organ(oid)s as pancreas or kidney should employs the same principles as in organogenesis relying on focal stimuli provided by MSC to organize other cell types in a proper manner. At this point our recent data describing heterogeneity in human MSC-derived CS provides a new outlook on this delivery mode and its potential impact on regeneration [81, 82].

In our study a CS from human adipose-derived MSC showed significant morphological heterogeneity with 2 distinct compartments of high and low density of cells ("hills" and "valleys respectively – see Figure 2, a-c). High-density regions were formed as a result of MSC proliferation cycles followed by coordinated contraction and migration of cells (see Supplementary video 1) which resembles a previously described contraction phenomenon that accompanies MSC-driven self-organization.

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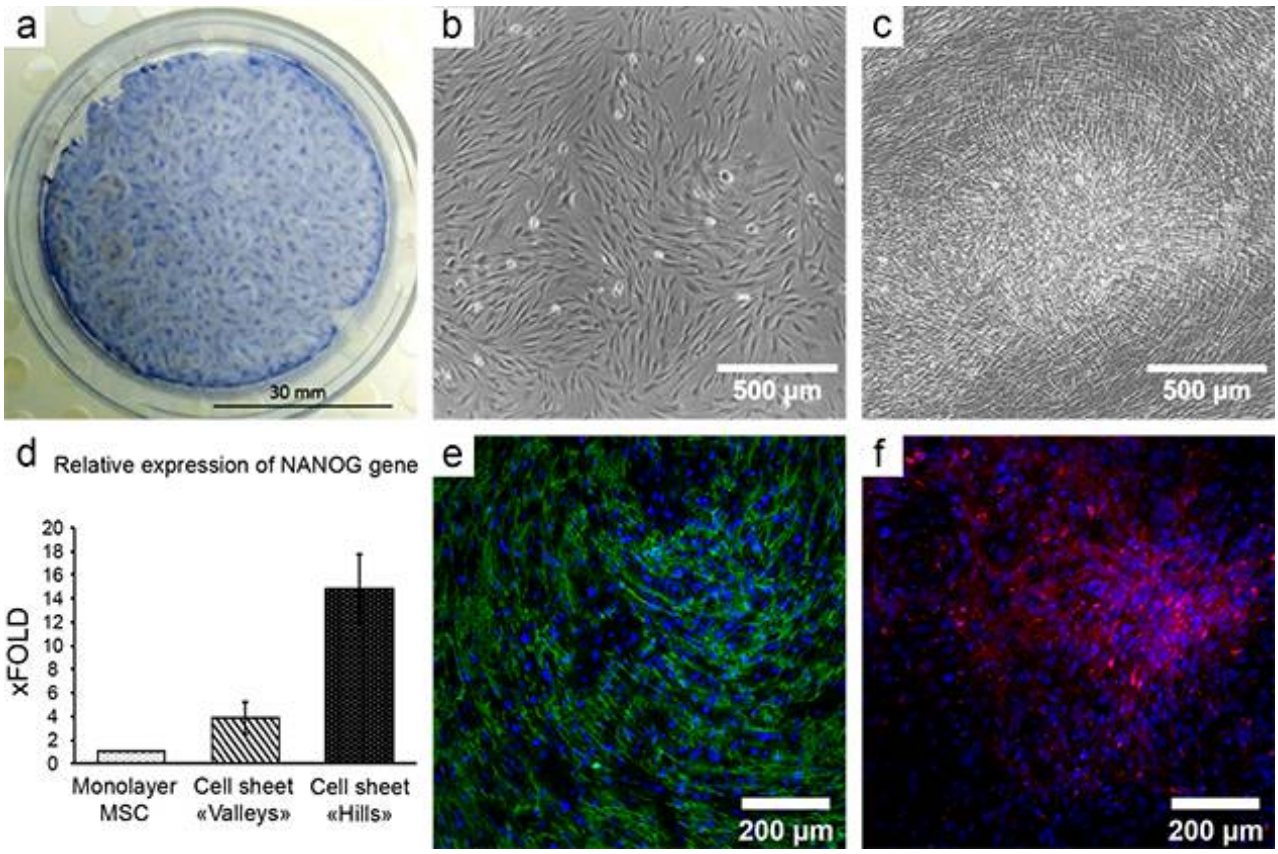

Figure 2. Self-organization of MSC in cell sheets results in formation of heterogeneous microenvironment.

a. Heterogeneous distribution of MSC in CS at day 12 of cultures; hematoxylin staining;

b-c. Microphotographs taken in the same field of view of MSC culture at day 1 (b) and day 12 (c). Formation of a high-density "hill" is observed as a result of MSC self-organization in CS; time-lapse microscopy, phase-contrast.

d. MSC subpopulations were picked by laser microdissection. MSC in the "hills" show increased expression of stemness-associated factor (NANOG); real-time qPCR (n=3); data expressed as mean ± standard deviation are shown.

e-f. MSC in CS organise EDA-fibronectin in regular network of fibres (green, panel e) while laminin (red, panel f) is deposited primarily in "hills", but not in "valleys" creating foci of ECM deposition in CS (f); immunofluorescent microscopy, nuclei are stained by DAPI (blue); from Nimiritsky et al. [81].

For details of experimental procedures please address supplementary Materials and methods online.

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Mesenchyme-mediated guidance in development relies on ECM differential distribution that has crucial importance as it facilitates attraction and retain of different cell types [77]. We found that in MSC sheets two ECM proteins – EDA-fibronectin and laminin are distributed uneven with higher density in "hills" and lower – in "valleys" (Fig. 2, e and f). Condensed laminin deposition was detectable only in "hills" of CS and in monolayer of MSC no acquirable signal was detected (data not shown). Laminin is known to mediate interaction of mesenchymal and epithelial cells leading to assumption that heterogeneity of its in CS reflects formation of guiding ECM stimuli that can orchestrate organization of endothelial, epithelial and mature stromal cells on this *in vitro* formed feeder.

Differential morphology and ECM deposition in CS suggest that different MSC subtypes main retained in them so we used laser microdissection to separate "hills" and "valleys" at day 12 to evaluate relative expression of stemness-related gene *NANOG* in MSC from different microenvironments.

We compared laser-dissected "hills" vs. remaining "valley" portion of CS and found significantly (up to 15-fold) increase of *NANOG* expression compared to "valleys" of same CS (Figure 2, d). It suggested that heterogeneity of CS results in either sorting of different cells to different compartments of the construct or local shift of stemness *in situ* resembling "mapping" function of mesenchyme known in development and suggesting that MSC sheets actually can be considered as a guiding construct rather than just delivery tool.

Previously MSC have been successfully used to generate pre-vascularized structures and endothelial cells spontaneously organized in tubes once were co-seeded with MSC for sheet formation [79, 83]. This provided a method for fabrication of tissue-like 3D structures by stacking pre-vascularized CS together without loss of nutrition inside the construct. Nevertheless, our data suggests more attention to potential use of MSC-based CS as an effective driver of organoid or tissue self-assembly as it possesses an important "mapping" trait required for organization of other cell types by gradients of stimuli (Fig. 3).

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Previous data obtained by our group and other authors using single cell analysis found numerous subpopulations in primary MSC with varying sensitivity to hormones, differentiation ability and signaling [84-86]. However, origin of stimuli gradients and CS heterogeneity is subject for elucidation taking into account data on mesenchymal cells' role in differentiation control during development and organogenesis *ex vivo*.

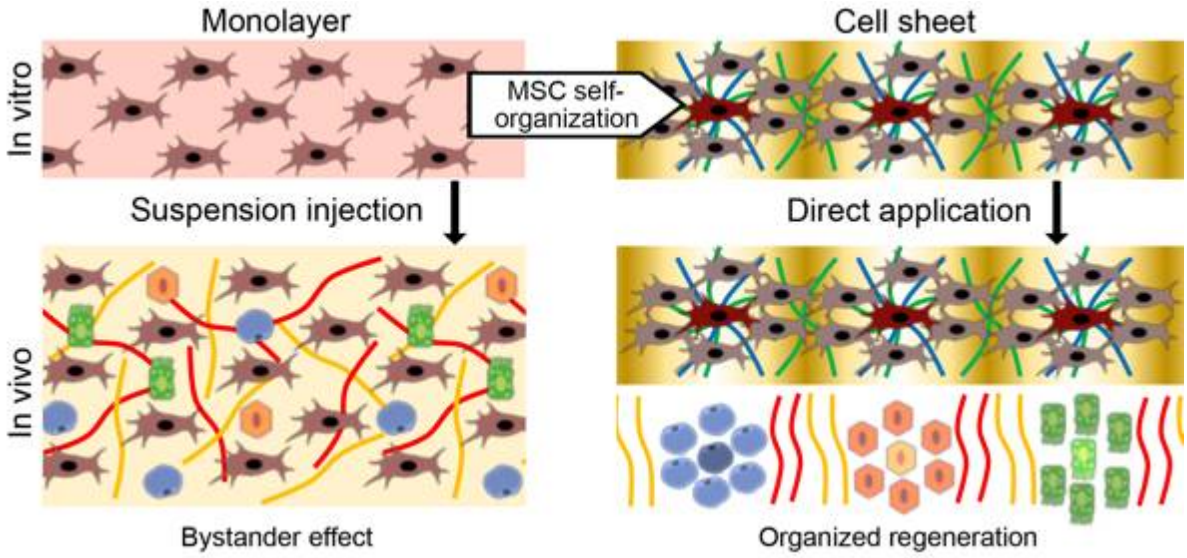

Figure 3. Self-organization of MSC as a basis of its organizing function in regeneration.

Conventionally used injection of MSC cultured in monolayer (left part) is limited to paracrine (bystander) effects of delivered cells. Self-organization during CS formation results in retain of microenvironment with gradient of stimuli that organize regeneration after delivery (right part).

7. Concluding remarks

Present paper reflects our vision on how stem cell delivery might be improved basing on knowledge of human development, system biology and – in particular – limitations of existing cell therapy approaches.

To our knowledge regeneration after injury has not been previously compared to the recovery of ecosystem structure. Terminology of ecological and evolutionary biology has been extensively used in cancer [87-89] and stem cell [90] biology, so

we consider our approach reasonable to explain importance of putative feeder formed in early stages of regeneration for its outcome.

In our view participation and recruitment of MSC during formation of feeder has physiological rationale. Our point is supported by "mapping" role of mesenchymal cells in development and by prominent examples suggesting that adult MSC retain this ability even *ex vivo* driving functional organoid formations and vascularization in a dish. The concept of feeder as a base layer for successful regeneration might be a subject for discussion yet we suggest it as a summary of our knowledge about regeneration and data from other groups.

This concept overall shifts our understanding of MSC-based CS as a delivery platform for regenerative medicine. We believe that CS is not only a mean of "cells + ECM" deployment, but may present a unique object for study of mammalian regeneration, organoid assembly or tissue modelling. MSC within CS may retain or reproduce certain traits of mesenchymal cells in development to orchestrate regeneration. Origins and consequences of heterogeneity we observed in CS as well as its influence on other cells (including tissue-specific progenitors and terminally differentiated cells) is a subject for further investigation.

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