3. Early tissue patterning is recreated by mouse embryonic fibroblasts in a three-dimensional environment

3.1. *Introduction*

Cellular self-organization studies have been mainly focused on models such as *Volvox*, the slime mold *Dictyostelium discoideum*, and animal (metazoan) embryos. Interestingly, these models have something in common: their individual cells need to adhere together to form a cohesive organism. Free *Dictyostelium* cells synthesize a sticky 24-kDa glycoprotein under nutritional deficit, becoming increasingly adhesive and promoting the formation of a cellular aggregate that undergoes differentiation into an organized structure [30-33]. A similar mechanism occurs in early animal embryos where cells adhere together to form the tissues and organs during development. Also animal tissues undergoing regeneration present intrinsic properties of embryonic systems including cell multipotential capacity, pattern expression of developmental genes by a self-organization process to rebuild tissue complexity and function [24, 34-38]. For instance, the process of mammal digit tip regeneration displays phases similar to those found in limb regeneration in amphibians: (a) Apical Epithelial Cap (AEC) formation, (b) blastema-like formation by dermal fibroblast and myotube dedifferentiation and (c) regeneration or re-development, leading to scar-less wound healing [24, 34-38]. We speculate that the recreation of a suitable microenvironment similar to that of regenerative areas (with reduced or non-inflammatory response) can be possible to develop *in vitro* by recreating the biological, biophysical and biomechanical conditions. This microenvironment would promote the intrinsic capacity of adult tissues to proceed to regeneration instead of scarring [10]. In particular, we are interested in obtaining *in vitro* systems that resemble some aspects of a regenerative blastema, where embryonic fibroblasts not only acquire properties such as cell dedifferentiation and multipotentiality but they also, as a whole, engage in a re-developmental-like program.

Zhang et al. developed in 1995 the self-assembling peptide RAD16-I, that forms promising three-dimensional scaffolds for tissue engineering applications. This peptide has been used to promote growth and proliferation of multiple cell types including chondrocytes, hepatocytes, endothelial cells, osteoblasts and neuronal cells as well as embryonic and somatic stem cells [12, 13, 39-44]. In a recent work, we demonstrated that only after culturing primary mouse embryonic fibroblasts (MEFs) into a three-dimensional self-assembling peptide scaffold for several days they up-regulated osteopontin (OPN) as well as two metalloproteinases, MMP-2 and MMP-9, known as type IV and V collagenases or the 72-kDa gelatinase A and 92-kDa gelatinase B, respectively, proper of an embryonic regenerative system [45, 46]. Moreover, only osteoinduced MEFs 3D-cultures were able to develop mineralized matrix by von Kossa staining but not 2D-cultures and 3D-culture controls. Alkaline phosphatase activity (ALP) was highly expressed in all 3D-cultures, indicating that the 3D-environment promotes ALP activity independently of the osteogenic conditions. Since the RAD16-I peptide scaffold does not contain any specific peptide-signaling motif, we define this environment as “non-instructive” from the point of view of cell receptor recognition/activation (either integrin- or growth factor-

like receptor), suggesting that the three-dimensional environment *per-se* promotes cellular responses. Finally, the system produced collagen type I and up-regulated the expression of the transcription factor Runx2, suggesting that the cells acquire osteoblast-like phenotype [47]. Additionally, other groups have shown previously that mesenchymal cells from bone marrow origin, mouse embryonic stem cells as well as mouse embryonic fibroblast can be differentiated into cartilage-, fat- and bone-like tissue but strictly under specific inductive media conditions [48-56].

The *in vitro* cellular system we describe here undergoes a process that resembles many aspects of animal development including cell aggregation, proliferation, migration and tissue specification but most importantly: morphogenesis and pattern formation.

3.2. *Results*

3.2.1. Fibroblasts suffer a morphogenetic process when cultured in RAD16-I

To understand the influence of the biomechanical and biological environments on the behavior of fibroblasts in the peptidic nanofiber scaffold RAD16-I, we varied both the concentration of peptide used to form the scaffold (0.25-0.5%) and the concentration of FBS (2-15 %) added to the media (**Figure 2 B**). Surprisingly, we observed that, when cultured in 0.25 % RAD16-I and 15 % FBS, fibroblasts engage in a morphogenetic process that renders a compact cell mass with a central axis and two thick parallel structures at both sides of the central line (**Figure 2 C**). During the first 1-2 days in culture the cells contracted the scaffold from a disk-shape to a much smaller flat dense disk (stage 1). 4-6 days after encapsulation, the edge of the disk continued the contraction compacting its perimeter and turning it into a wheel-like shape with a semicircular cross-section or dome shape (stage 2). Next, between day 7 and day 15 two diametrically opposite zones at the edge of the wheel or dome started actively contracting inward converging at the center, compressing both sides (stage 3). As a consequence, the compaction of the cell masses from each side of the dome caused a merging zone that forms a “middle line”. This process seems to elongate the body along the axis producing two large and dense paraxial structures, which results in a 3D-bilateral assembly 11 to 15 days after encapsulation (Stage 4) (**Figure 2 C**). At this point the cell mass has gone through the main morphological changes which can be separated in the four stages cited above. For better understanding, we show a schematic representation suggesting the main morphological process that the system undergoes to develop into a 3D-bilateral structure (**Figure 2 D**). Interestingly, optical cross-sections at two time points suggested the formation of an internal cavity as a result of this morphogenetic process (**Figure 2 C**). The final shape presents bilateral symmetry (3D-bilateral) and resembles some aspects of a vertebrate embryo undergoing axis formation.

The development into a bilateral structure happens in more than 90 % of the cases estimated after performing independent experiments in 3 different laboratories in Cambridge (USA), Leipzig (Germany) and Barcelona (Spain). We discovered that the quality and origin of serum has a huge effect on the success of the morphogenetic process to the point that certain sera are unable to trigger it. Moreover, we noticed that heat-inactivated as well as UV-inactivated sera were useless to induce contraction to MEFs. Therefore we assumed that some specific growth factors in the sera were key in this phenomenon.

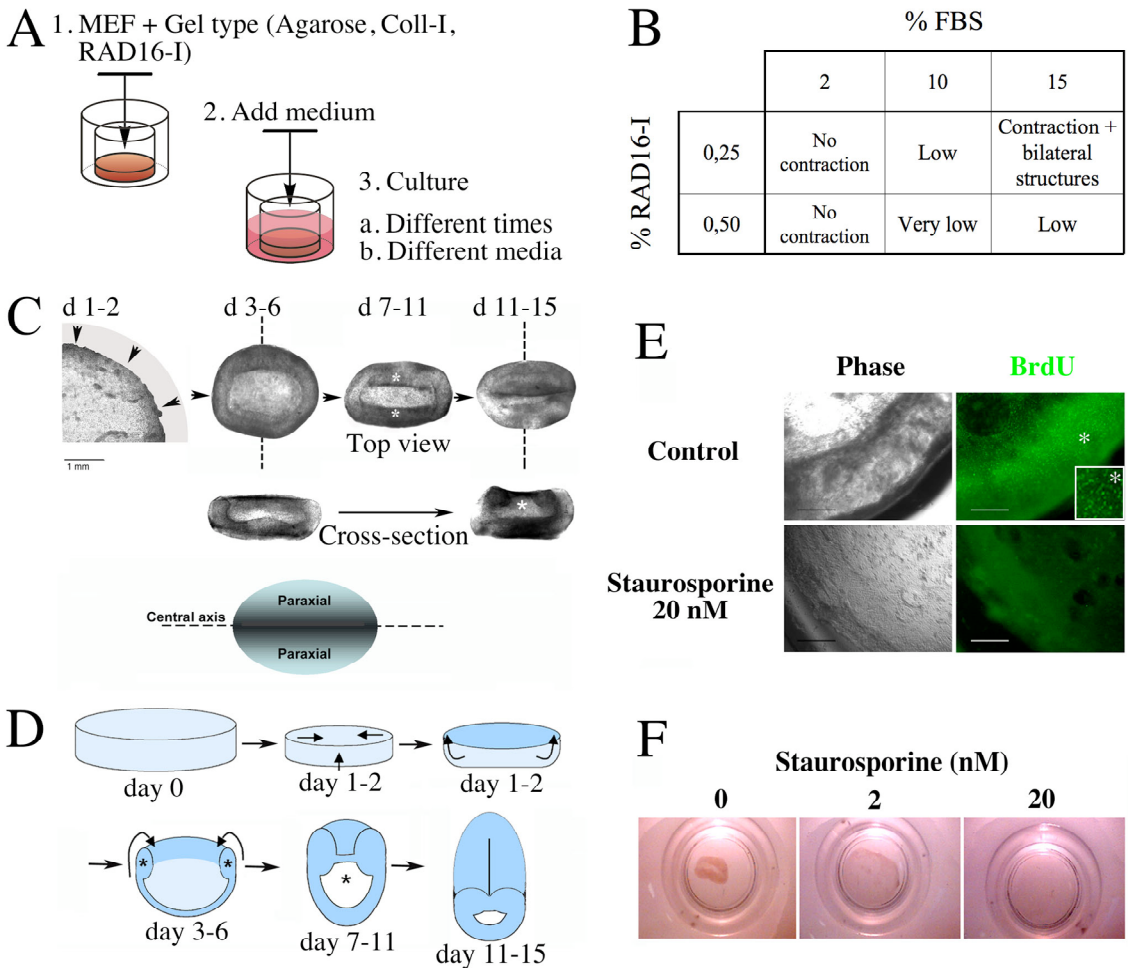


Figure 2: Morphogenetic changes of Mouse Embryonic Fibroblasts in 3D cultures.
(**A**) Scheme of a MEF culture and differentiation protocol. (**B**) MEFs were cultured using two different concentrations of RAD16-I to form hydrogels with different stiffness. They were cultured with three different concentrations of FBS in the FM formulation. Rate of contraction was observed, for each combination of FBS percentage and RAD16-I concentration, at day 10. Results are presented qualitatively in a Table. (**C**) Main morphological process observed by visual inspection of MEFs cultured with FM in RAD16-I 0.25%. The figure shows the contraction of MEFs in the hydrogel. In 7-15 days a 9 mm diameter hydrogel disk becomes a compact tissue-like cell mass of about 2 x 4 mm with bilateral patterning. Optical cross-sections depict the presence of an internal cavity. Asterisks at both sides of the structure indicate the zone of lateral contraction forces. (**D**) A model indicating the main morphological processes is presented as a guide to help understanding the development of the 3D-bilateral structure. The asterisks indicate also the zone of lateral force generation and the presence of a cavity developed during the morphological process and the empty cavity present in the structure. (**E**) Inhibition of the 3D-bilateral structure development by cell cycle arrest induced by staurosporine. MEFs were incubated with and without staurosporine (20 nM) during 7 days. Proliferation was studied by means of a BrdU pulse followed by immunostaining against BrdU. Asterisk shows a close-up of the positive stained MEFs. Bar = 500 μm. (**F**) MEFs were incubated, during 14 days, with different concentrations of staurosporine (0, 2, 20 nM) to further analyze the effect of proliferation in the contraction phenomenon. Bar = 500 μm.

In order to normalize and improve the success of this morphogenetic event we tested the addition of growth factors naturally present in sera such as PDGF-BB, IGF-I, VEGF and TGF-β. Results show that concentrations of 0.67 nM PDGF-BB and 0.02 nM TGF-β are important for MEFs so they can proliferate and engage in the morphogenic contraction. Specifically, 0.004 nM TGF-β can already induce symmetry (**Figure 3**), but is not enough to complete the morphogenetic process. Then, 0.012 nM TGF-β is capable of inducing morphogenesis (**Figure 3**) but has low success in this task (results not shown). TGF-β concentrations of 0.02 nM or more induce morphogenesis in almost all experiments (93 % success in this thesis and even higher if we include experiments of other laboratories).

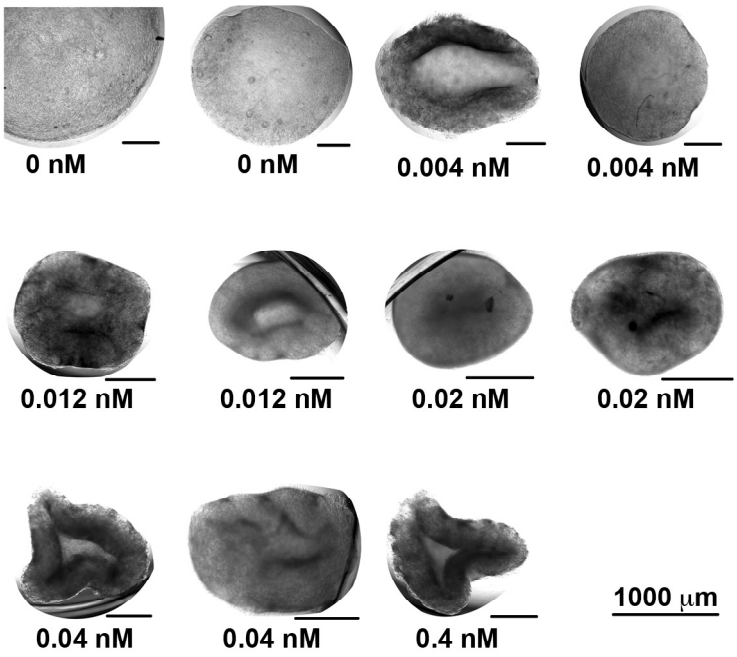
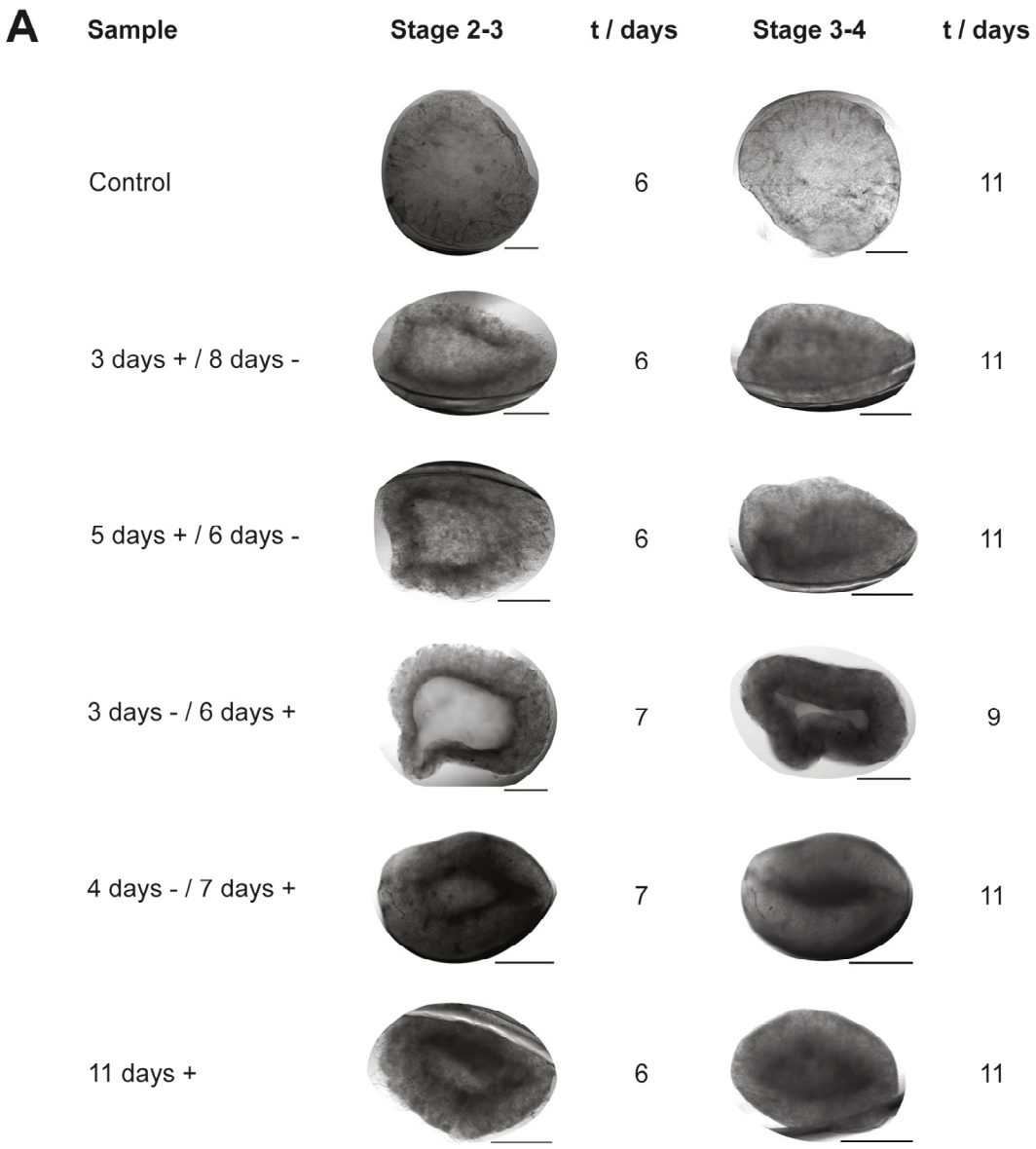


Figure 3: Effect of TGF-β concentration on the morphology of the 3D culture of MEFs. MEFs were cultured in RAD16-I in regular culture conditions (see materials and methods) with different concentrations of TGF-β in the culture medium, which are indicated under each sample. The pictures show the final morphologies of these cell cultures when there was no more significant progression in the morphological changes. All bars represent 1000 μm.

Apart from the concentration of TGF-β required for the morphogenetic process, we evaluated if TGF-β was necessary during all the experiment or if it was only required at a particular moment of the culture protocol. We used TGF-β only at the first 3 or 5 days of culture and then progressively eliminated the TGF-β from the culture medium. Parallelly we deprived other MEFs from TGF-β for the first 3 or 4 days and then added TGF-β to the medium. The morphology of MEFs was not affected by these variants in the supplementation profile of TGF-β (**Figure 4**), so any of these culture protocols might be used to generate symmetry in MEFs 3D cultures.


A
Sample
Stage 2-3
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Stage 3-4
t / days
Control
6
11
3 days + / 8 days -
6
11
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6
11
3 days - / 6 days +
7
9
4 days - / 7 days +
7
11
11 days +
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B
[TGF-β] vs. time
[TGF-β] / nM
0.02
0.015
0.01
0.005
0
0
2
4
6
8
10
12
time (days)
Control
3 days + / 8 days -
5 days + / 6 days -
3 days - / 6 days +
4 days - / 7 days +
11 days +

Figure 4: Effect of the TGF-β supplementing profile on the morphology of the 3D culture of MEFs.
This experiment was designed to find if TGF-β was required at any particular time point of the culture protocol in order to trigger the formation of a symmetrical axis. We were interested in finding if TGF-β was necessary from the very beginning or if it was required during all the culture time. We cultured MEFs in regular 3D culture conditions for MEFs in RAD16-I (see materials and methods) but varying the supplement of TGF-β during the culture. We used culture of MEFs with and without TGF-β as positive and negative controls and tried four different variants in the supplementation of TGF-β. Each of these variants consisted of first some days with or without TGF-β and then progressively decreasing or increasing TGF-β concentration in the culture medium. (**A**) Table that shows pictures of two different time points of each of the variants of TGF-β supplementation. All cultures that have a supplement of TGF-β at some point seem to suffer similar morphological changes, so the moment when TGF-β is supplemented seems to induce no significant changes in the generation of a symmetrical axis. (Bars under each picture represent 1000 μm). (**B**) This graph shows the theoretical concentration of TGF-β in the culture medium of each variant after culture media replenish as function of the culture days.

Finally, IGF-I and VEGF have also been tried as supplements to the culture medium and seem to have no significant effect on tissue contraction. The fact that TGF-β and PDGF-BB are necessary for the contraction of this tissue and the appearance of symmetry is consistent with other investigations that show how PDGF and TGF-β enhance fibroblast-mediated collagen gel contraction [57, 58]. In fact, we eventually observed the contraction of collagen-I gels mediated by MEFs (**Figure 5 G,H**), although these contractions were not accompanied by the formation of a bilateral structure. Moreover, on the one hand PDGF-A has shown to promote chondrogenesis at early stages of limb development [59], which, in some way, may be related to the effect that PDGF-BB exerts on the system described here. On the other hand TGF-β is a well-known growth factor that mediates mesenchymal condensation [60], a process that shares many similarities with the contraction phenomenon here described.