

Figure 5: Osteogenic and chondrogenic differentiation of MEFs cultured in RAD16-I, Collagen type I and Agarose

MEFs were cultured in Fibroblast Medium (FM) (Samples **B**, **E**, **F**, **G** and **J**), chondrogenic medium (Samples **A**, **C** and **D**) and osteogenic medium (Samples **H**, **I** and **K**) as explained in text. Agarose (**A**, **B**, **I** and **J**), collagen-I (**D**, **E**, **G**, **H** and **K**) as well as RAD16-I (Samples **C** and **F**) were used as 3D environments for MEFs. Toluidine blue staining was used to assess the synthesis of GAGs in samples **A-F**. It

can be observed that MEFs cultured in RAD16-I express GAGs when cultured with (**C**) or without chondrogenic supplements (**F**). Von Kossa staining was used to identify osteogenic commitment in MEFs in the mentioned 3D environments (Samples **G-K**). It can be observed that calcium precipitation is observed in all samples treated with osteogenic medium (**H**, **I** and **K**), but not in samples cultured with Fibroblast Medium (**G** and **J**).

3.2.2. Proliferation is required to complete the morphogenetic changes

To further understand this phenomenon, fibroblasts, cultured as explained above, were treated with Staurosporine (an inhibitor of the Protein Kinase C, that arrests cells in G1). Staurosporine clearly abrogated the development of the 3D-bilateral structure. **Figure 2 E** shows that no evident incorporation of BrdU was detected in Staurosporine-treated samples, while control samples exhibit high proliferation in the outermost circle of the cell mass. Moreover, a dose-response inhibitory effect on the 3D-bilateral structure development can be observed when diluted concentrations of Staurosporine are added to the cultures (**Figure 2 F**). These results indicate that during this morphogenetic event the cell density increases not only by contraction but also because of proliferation, suggesting that the system needs to reach a critical cell mass for this phenomenon to occur. This result strengthens the conclusion that PDGF-BB and TGF- β may affect the morphogenic process by enhancing cell proliferation and condensation, which is one of their known downstream effects [61].

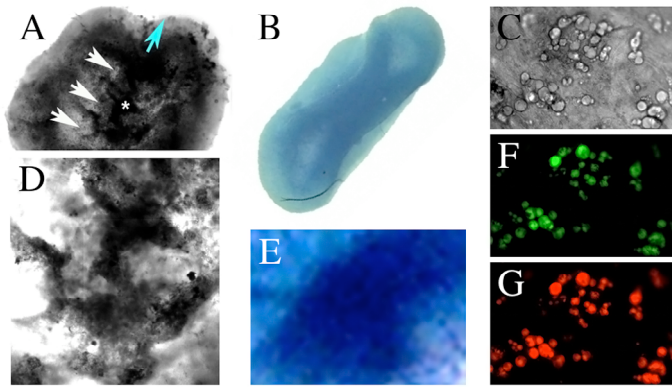


Figure 6: Multipotential capacity of Mouse Embryonic Fibroblasts.

(A-J) Phenotype of MEFs cultured in 3D-self-assembling peptide hydrogel RAD16-I was assessed under different culture conditions. (A, D) After osteogenic induction, von Kossa staining revealed calcium deposition within the cell cultures. (B, E) Toluidine blue staining of MEFs showed default chondrogenic differentiation in control cultures, without any need of supplements. (C) Adipocytes in non-supplemented cell cultures were detectable by visual inspection. (F, G) Nile red staining stained the neutral lipids of these adipocytes (F, fluorescein filter, green; G, rhodamine filter, red).

H

Medium	Osteogenesis		Chondrogenesis		Adipogenesis	
	FM	Osteo	FM	Chondro	FM	Adipo
2D	-	-	-	-	-	-
RAD16-I	-	+++	+	++	+	++
Collagen	-	+++	-	-	+	+
Agarose	-	+++	-	-	+	+

Pictures of differentiation results in 2D-cultures Controls and 3D-cultures in agarose and collagen type I are presented in **Figure 5**. (H) Osteogenic, chondrogenic and adipogenic differentiation assays were performed as indicated on MEFs and three different stainings were used to identify cell commitments: Von Kossa staining was used to identify osteogenic, toluidine blue staining for chondrogenic and Nile red staining for adipogenic differentiations. For reference level of differentiation: - (negative), + (slightly positive), ++ (positive), +++ (highly positive). The table shows that MEFs can acquire osteogenic and adipogenic phenotypes in three dimensions, but only in RAD16-I they can undergo natural chondrogenesis.

3.2.3. Fibroblasts acquired multipotential capacity after culture in RAD16-I gels

Fibroblasts have been shown before to have multipotential capacity into mesenchymal lineages [45, 47-55]. To further assess the multipotentiality of MEFs we evaluated the osteogenic, adipogenic and chondrogenic capacities of fibroblasts cultured in the RAD16-I nanofiber scaffold, in agarose (a non-instructive 3D environment) and in collagen-I (the natural extracellular matrix of dermal fibroblasts and bone). We questioned us whether or not these three chemically unrelated matrices promote the acquisition of mesenchymal potentiality as observed before in RAD16-I [45]. Osteogenic, adipogenic and chondrogenic phenotypes were assessed by Von Kossa, Nile red and toluidine blue staining, respectively. **Figure 6H** shows that fibroblasts undergo acute osteogenesis in all 3D environments after induction with osteogenic supplements, which is evidenced by the high extracellular deposition of calcium salts (**Figure 6 A,D** and **Figure 5 G,H,I,J** and **K**). Osteogenesis only occurred with addition of osteogenic supplements, whereas adipogenesis happened with (not

shown) and without adipogenic supplements (**Figure 6 C,F and G**). More interestingly, chondrogenesis arose only in the self-assembling peptide scaffolds, with (**Figure 5 C**) and without chondrogenic supplements (**Figure 6 B, E and Figure 5 F**), indicating that there's some relationship between chondrogenesis and the biomechanical and non-instructive characteristics of RAD16-I.

Western blot analysis revealed that the pre-osteogenic transcription factor Runx2 was upregulated in MEFs cultured without inductive supplements in the nanofiber scaffold RAD16-I, comparing to expression levels of the same cells cultured in 2D (**Figure 7 A**). Then, the addition of differentiating supplements caused an upregulation of the expression of Runx2 in 2D cultures, while in 3D cultures Runx2 remained constant. Runx2 is a transcription factor present in hypertrophic chondrocytes (which only mineralize the ECM when osteochondral ossification begins [62, 63]) and normally during osteogenesis [64]. In our system mineralization only happens in 3D cultures in the presence of osteogenic supplements. Moreover, the expression of Runx2 is upregulated in 3D cultures with and without osteogenic induction, suggesting that, in our system, fibroblasts develop by default all the way to hypertrophic chondrocytes but not to osteoblasts. This implies that other factors known to induce osteogenesis in hypertrophic chondrocytes are not present in our system (for instance the vascular cross-talk signaling). Interestingly, 2D cultures of MEFs induced with osteogenic factors upregulated the expression of Runx2 but failed to mineralize the matrix, suggesting that in the 3D system, besides the upregulation of Runx2 there must be other factors that determine the osteogenic commitment.

3.2.4. Default chondrogenic commitment of MEFs cultured in RAD16-I gels

First, we analyzed by Real-time RT-PCR the presence of the transcription factor Sox9, a key player in the formation of cartilage during development [62, 65-68], and its downstream targets Sox5 and Sox6. Sox9 is required for the expression of Sox5 and Sox6 and these three SRY type transcription factors (the Sox trio) are then expressed until chondrocytes enter hypertrophy [65]. The expression of Sox9 presents a pattern of expression with two peaks of upregulation, one at day 3 (Stage 1) and the second at day 11 (Stage 4), separated by a downregulation zone at day 7 (Stage 3) (**Figure 7 D**). These changes in expression correlate well with the morphogenic changes previously described. For instance, at day 3 (stage 2) the structure contraction is isotropic continuing until day 6 (stage 3) (**Figure 2 C**). Interestingly, at day 7 (stage 3) the structure starts bilateral contractions suggesting a dramatic change in the morphogenic process that might affect the expression of Sox9. At day 11 (Stage 4) the morphogenetic changes finish and the bilateral structure up-regulates Sox9 again but localized at the paraxial zone (**Figure 9 D**). In 2D cultures Sox9 upregulation was less intense with a gradual increase over time (**Figure 7 D**).

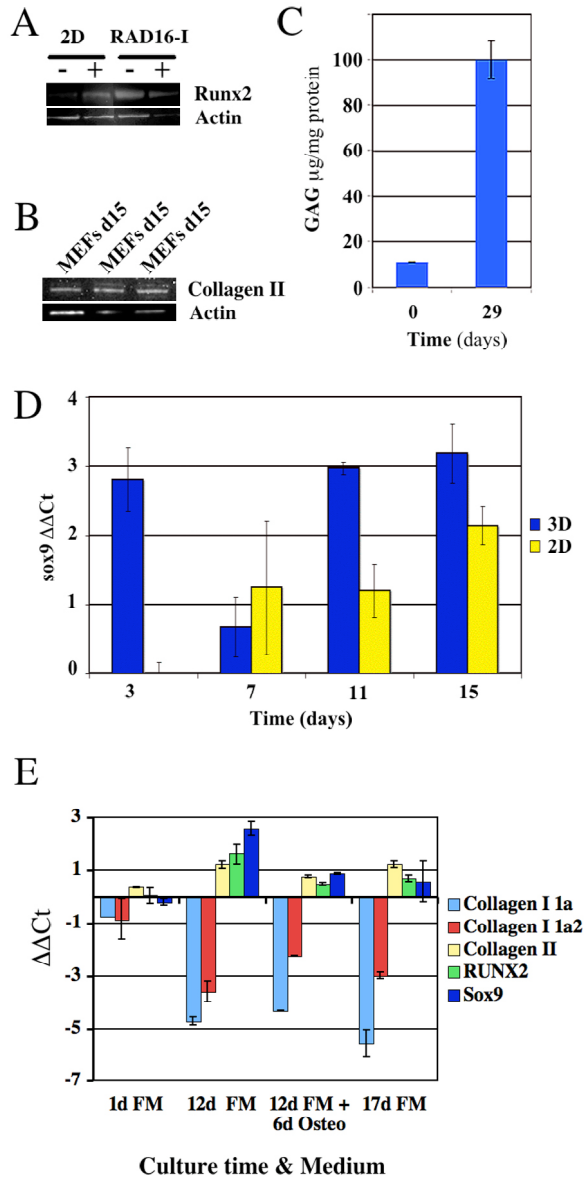


Figure 7: Molecular characterization of the mesodermal induction process.

(A) Runx2 expression was analyzed by western blot in MEFs cultured in RAD16-I after the osteogenic differentiation protocol previously described. 2D cultures were used as control. (B) Collagen type II was analyzed by western blot in MEFs cultured in flask cultures (2D) after 1 and 15 days in culture and in RAD16-I (3D) during 15 days in culture with Fibroblast Medium (FM). (C) Glycosaminoglycans (GAGs) from 3D-self assembling peptide cultures of MEF at days 0 and 29 were quantified with DMMB (1,9-dimethyl-dimethyleneblue) using chondroitin sulfate as standard. (D) Real Time RT-PCR of Sox9 transcription factor. Total mRNA was isolated from MEFs cultured in RAD16-I with FM, and from 2D controls, after days 3, 7, 11 and 15. (E) Real Time RT-PCR of Collagen I, Collagen II, Runx2 and Sox9. Total

mRNA was isolated from MEFs cultured in Fibroblast Medium during 1, 12 and 17 days and cultured 12 days in Fibroblast Medium followed by 6 days in osteogenic medium.

It is known that Sox9 directly controls the expression of two other chondrogenic transcription factors, Sox5 and Sox6 [65, 66, 69]. Hence, we checked whether the expression of the transcription factor Sox9 had the very same effects it has in a developing embryo. So we quantified the expression of Sox5 and Sox6 in MEFs cultured in 3D with or without supplemented TGF- β . On the one hand, we found that the expression of Sox5 and Sox6 is greater when MEFs are cultured without TGF- β (Figure 8). In fact, repeatable and higher levels of production of Sox5 and Sox6 were only detected in RNA extracts from MEFs cultured without TGF- β . MEFs cultured in 2D with regular FM medium and in 3D with medium supplemented with TGF- β have similar levels of expression of Sox5, both of them around 6.5

times lower than that of MEFs cultured in 3D without the supplement of TGF- β . Moreover, Sox6 was only detected in MEFs cultured in 3D without TGF- β (**Figure 8**). On the other hand, MEFs cultured without the supplement of TGF- β have a morphological aspect very similar to stage 2 of the morphogenetic process that suffer MEFs cultured with TGF- β . Therefore, we can infer that MEFs cultured in 3D without TGF- β represent stage 2 of the morphogenetic process we describe and MEFs cultured with TGF- β for the same period of time represent stage 4 of this same phenomenon. Bringing these two ideas together allow us to hypothesize that Sox5 and Sox6 are upregulated at stage 2 of the morphogenetic phenomenon and no significant expression of Sox5 or Sox6 is detected at stage 4 of contraction.

To further understand the gene expression of the 3D system during default chondrogenesis and after osteogenic induction, we evaluated the expression of the transcription factors Runx2 and Sox9, as well as ECM proteins such as collagen type Ia1, collagen type Ia2 and Collagen type II using real-time RT-PCR. Results show that at day 12 (stage 4), when the 3D-bilateral structure closes, Sox9, Runx2 and Collagen type II are over-expressed compared to the expression levels of MEFs cultured in flasks (**Figure 7 E**). At the same time, the synthesis of Collagen type I is strongly down-regulated (**Figure 7 E**), meaning that MEFs cultured in RAD16-I gradually lose their fibroblastic phenotype (characterized by the synthesis of Collagen I) and become chondrocyte-like cells (expressing Collagen type II, Runx2 and high levels of Sox9). Then, 5-6 days after osteogenic induction, the expression levels of Collagen type II, Runx2 and Sox9 decrease (**Figure 7 E**). Collagen type II remains constant after 17 days of culture without osteogenic supplements as showed by Real Time PCR (**Figure 7 E**) and Western Blot analysis (**Figure 7 B**), giving more evidence to the default chondrogenic commitment of MEFs cultured in RAD16-I. The downregulation of Sox9 and Runx2 appear to be independent of the presence of the osteogenic induction (**Figure 7 E**). In the case of Collagen type I, in presence of osteogenic induction an upregulation can be observed compared to conditions without differentiating factors (**Figure 7 E**).

A

Sample	LP		Sox5		Sox6	
	ΔCt	$-\Delta\Delta Ct$	ΔCt	$-\Delta\Delta Ct$	ΔCt	$-\Delta\Delta Ct$
2D	19,22		17,71		23,07	
	21,13		17,73		No amp	
	No amp		18,73		No amp	
TGF- β -	17,34	1,88	15,22	2,49	21,41	1,66
	20,07	1,06	15,33	2,4	18,26	
	23,105		15,5	3,23	20,47	
TGF- β +	17,59	1,63	17,86	-0,15	No amp	No amp
	20,43	0,7	17,52	0,21	No amp	No amp
	23,25		18,19	0,54	20,97	

B

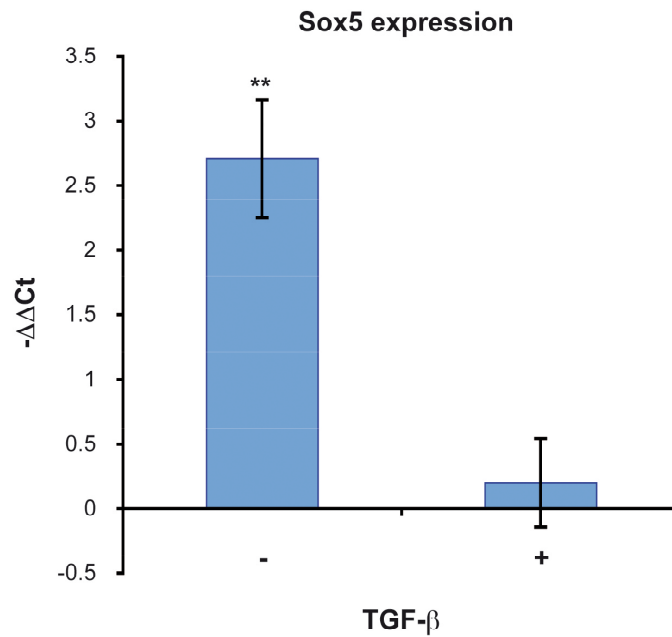


Figure 8: Expression of Sox5, Sox6 and Link Protein in MEFs cultured with and without TGF- β .

MEFs were cultured in RAD16-I with and without TGF- β until MEFs cultured with TGF- β reached stage 4 of morphogenesis (between 9 and 14 days). RNA was extracted from these 3D cell cultures and, for internal reference, from 2D cultures of the MEFs from which 3D experiments were prepared. RNA was only used when A_{260} / A_{280} and A_{260} / A_{230} were between 2.0 and 2.2 for quality insurance. **(A)** One-step qPCR using a Corbett thermocycler showed the expression of Sox5, Sox6 and Link Protein in terms of ΔCt and $-\Delta\Delta Ct$ in MEFs cultured with or without TGF- β , using 2D MEF cultures' gene expression as internal references. **(B)** The graph shows $-\Delta\Delta Ct$ values of Sox5. **Expression of Sox5 in MEFs cultured without TGF- β is higher than that of MEFs cultured with TGF- β ($p < 0.05$).

Cartilage-like tissues are characterized by an extracellular matrix rich in proteoglycans [70] so we analyzed the presence of link protein, a component of the proteoglycans responsible for stabilizing the link between Aggrecan molecules and Hyaluronan polymers [67]. On the one hand, Real time PCR analysis has showed that link protein is upregulated in MEFs cultured in RAD16-I in comparison to MEFs cultured in 2D (**Figure 8**). On the other hand, the expression of link protein seems to be dependent of the morphogenetic process, as there is significant difference in relative link protein gene expression between MEFs that contract (cultured in a high concentration of TGF- β) and MEFs that do not contract (cultured in a low concentration of TGF- β). To compare link protein gene expression of MEFs in these two different conditions, we cultured MEFs with and without TGF- β as supplement and isolated RNA from all the samples when cells cultured in medium with TGF- β were at stage 4 of contraction. One-step Real Time PCR rendered the results summarized in **Figure 8**. The expression of link protein in 3D samples cultured without TGF- β has significant difference with respect to the 3D samples cultured in TGF- β or the 2D ones ($p < 0.02$ and $p < 0.01$ respectively). Assuming that 3D cultures without TGF- β mimic stage 2 of contraction, this result indicates that MEFs at stage 2 of contraction produce cartilage-like ECM and that the production of link protein is dependent from the morphological process we described in this work. MEFs at stage 4 have shown no significant difference in the expression of link protein compared to the MEFs cultured in 2D.

Finally, we also evaluated whether MEFs synthesized glycosaminoglycans, another component of the cartilaginous ECM, when they were cultured in RAD16-I with Fibroblast Medium (FM) until stage 4 of contraction, confirming the default cartilaginous commitment of these fibroblasts (**Figure 7 C**). Moreover, when we stained the 3D-bilateral structures with Toluidine blue for glycosaminoglycans (GAGs), two positive zones parallel to the central axis were detected suggesting the development of a paraxial cartilage-like tissue (**Figure 9 C**). These results confirm the presence of a glycosylated proteoglycan, which could be aggrecan or perlecan. This last one is more consistent with the appearance of cartilage during early development [71] and the fact that there is no significant synthesis of link protein in MEFs at stage 4 of contraction.

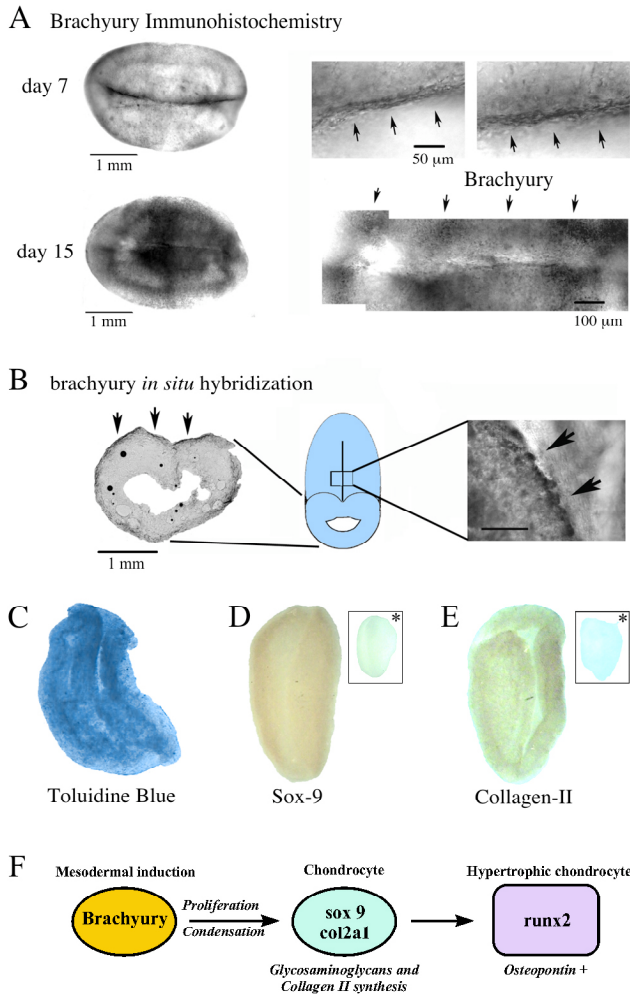


Figure 9: Morphogenesis correlates with pattern formation.

(A) Brachyury immunostaining of MEFs cultured in RAD16-I with FM for 7 and 15 days. Whole mount immunostaining showed the localization of Brachyury expression in the 3D-bilateral structures of 7 days and 15. Pictures on the right represent a magnification of the zones where brachyury is expressed for better view of the stainings. Brachyury is first expressed in the central axis and then in the paraxial zones. Clusters of expression can be observed at this moment (day 15).

(B) *In situ* hybridization was also performed to observe the localization of Brachyury. Totally contracted samples (15 days of culture in FM) were fixed and cryosectioned as indicated. *In situ* hybridization over a 14 µm slice of the 3D-bilateral structure showed that brachyury is expressed in the external paraxial zone. Short after closure of the central axis (11 days of culture), the cell mass was fixed. Whole mount *in situ* hybridization showed the localization of Brachyury within the cell mass.

(C-E) Toluidine blue staining and immunostaining for Sox9 and Collagen II were performed on MEFs cultured during 15 days in FM. Asterisks show control samples immunostained using only the secondary antibody HRP conjugated. These three stainings show that chondrogenesis occurs only in the two paraxial zones next to the symmetrical axis. (F) Finally, we propose a mesodermal commitment and cartilage-like tissue development model in our system based in the molecular markers detected.

3.2.5. Pattern formation accompanies symmetry appearance

To provide more insight into the possible patterned chondrogenesis, we studied the expression of brachyury, one of the first mesodermal-specific transcription factors that are expressed in the presumptive notochord and in the early mesoderm [72-74]. In different culture times, Brachyury stained positive by immunohistochemistry first in the central axis (day 7), and then (day 15) in the entire paraxial zones of the 3D-bilateral structure (**Figure 9 A**). The expression of Brachyury was confirmed by regular RTPCR (**Figure 10**) and its localization was visualized by *in situ* hybridization. Probes were prepared with the same primers used for regular RT-PCR and after the staining we observed that the Brachyury mRNA was mainly expressed at the same zone detected with immunohistochemistry, confirming the expression of this early organizer transcription factor in the 3D-bilateral structures (**Figure 9 B**). Staining of a cross-section of this structure depicts the clear presence of an internal cavity, observed before in **Figure 2**, and the expression of Brachyury at the external paraxial zone (**Figure 9 B**).

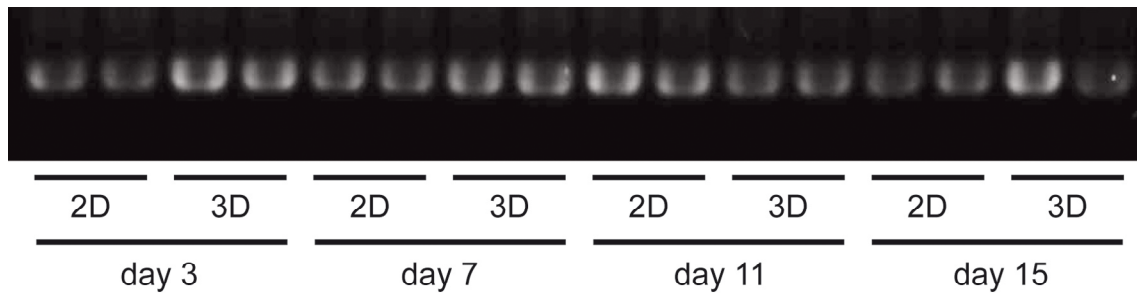


Figure 10: Brachyury analysis by RT-PCR

Expression of brachyury was confirmed by RTPCR in MEFs cultured in RAD16-I and in two dimensions at different time points (days 3, 7, 11 and 15). The agarose gel shows that MEFs express Brachyury when cultured both in 3D and 2D environments during the first 15 days of culture.

Furthermore, it is known that organized chondrogenic commitment is preceded by the expression of some set of transcription factors, such as Sox9. Thus, we studied the presence and localization of the transcription factors Sox9, a key transcription factor in early chondrogenesis [66], and immunostaining showed that Sox9 is mainly expressed in the paraxial zones next to the symmetrical axis (**Figure 9 D**). Finally, the synthesis of the key ECM component of cartilage Collagen II was assessed by immunostaining of the 3D bilateral structures cultured during 15 days in Fibroblast Medium (FM). Results showed that Collagen type II is expressed also at the paraxial zones next to the central symmetrical axis (**Figure 9 E**).

3.3. Discussion

Fibroblasts are known to be responsible for many regenerating events. A significant example is the regeneration of amputated limbs in amphibians, where fibroblasts generate a set of undifferentiated cells called blastema that are capable of proliferating and rebuilding the new limb [24, 34, 37, 38, 75]. Previously, fibroblasts cultured *in vitro* have also shown multipotential capacity [45, 52], and have also reproduced the mesenchymal condensation in micromass cultures [76, 77]. Nevertheless, it is still not clear what mechanism drives fibroblasts to this multipotential state and which is the exact phenotype of these undifferentiated fibroblasts [24, 34]. We hypothesize that the biomechanical as well as non-instructive properties of their environment are key for fibroblasts to have the ability to acquire a more undifferentiated phenotype that allows them to render most of the cell types of the mesodermal germ line.

Our work suggests that any 3D environment, under the culture conditions tested, and regardless of its chemical nature (self-assembling peptide nanofiber, polysaccharide, or protein matrix), is sufficient to promote dedifferentiation of MEFs into a multipotent mesenchymal progenitor-like cell (**Figure 5** and **Figure 6**). This opens the possibility that the cells “sense” the environment, reprogramming them into a multipotent progenitor. Osteogenic commitment of this progenitor cell in 3D environments occurs only after an osteogenic differentiation protocol, which is evidenced by the deposition of Ca^{2+} salts (**Figure 6 A** and **D**) and the expression of Runx2 (**Figure 7 A**) (also known as core binding factor, Cbfa1 and Osf2), positively regulates the expression of specific genes during early osteoinduction in hypertrophic chondrocytes, suggesting that these cells have acquired such a phenotype. Nevertheless, the specific characteristics of the 3D environment, such as the biomechanical and non-instructive properties, as well as the permissiveness toward cell-to-cell interactions, will provide cells with more or less multipotential capacity. This is clear in the case of RAD16-I, where cells acquired chondrogenic potential by default, as well as the unexpected morphogenetic process here described, suggesting that, in this case, the unique conditions obtained allowed the cells to proceed into a more-organized or emerging state: cellular self-organization. We cannot exclude that diffusion and oxygen gradients may play an important role in the morphogenetic process. It is not casual that the final wall thickness of the 3D bilateral structure does not exceed approximately 500 μm , which is the diffusion limit of oxygen in non-vascularized tissues. Moreover, we also speculate that the low oxygen concentration at the innermost part of the structure could favor the development of paraxial cartilage-like tissue. Notwithstanding this, the gene and protein expression profiles that accompany this morphogenetic event strongly suggest that there is a certain patterning involved.

For instance, these fibroblasts undergo spontaneous adipogenesis in 3 dimensions (**Figure 6 C, F** and **G**), but, only in the self-assembling peptide, MEFs begin a default chondrogenic differentiation process, presumably by creating a special cell microenvironment (**Figure 6 B**,

E and **Figure 9 C**). This could be due to the chemical differences between these three scaffolds. The polysaccharide (agarose) has shown low interaction with fibroblasts as well as poor contracting capacity: cells adopt a spherical shape and have very little movement inside the scaffold (**Figure 5**). Instead, collagen type I is the natural ECM component of the dermis and bone, ensuring high interaction between the cells and the matrix. Furthermore, this material could be instructive in guiding embryonic fibroblasts into these lineages, preventing them from spontaneous differentiation into a chondrogenic lineage (**Figure 6 H**). Finally, viscoelastic studies with RAD16-I (at different concentrations) over the years have shown that RAD16-I peptide concentrations between 1.7 and 2.8 mg/ml have a G' (elastic modulus) of 120 to 400 Pa, respectively. In our system we used final peptide concentrations of 2 mg/ml or 2.5 mg/ml, with a G' close to 370 Pa, a similar G' to that of extracellular collagen type I matrices, suggesting that the microenvironment in RAD16-I gels is mechanically similar to the natural ECM of fibroblasts. Therefore, as self-assembling peptide scaffolds lack inherent instructive capacity and have the same biomechanical properties as natural fibroblast ECMs, the default chondrogenesis here described could mean that Mouse Embryonic Fibroblasts can naturally undergo chondrogenic lineage differentiation at the mentioned culture conditions (**Figure 6 H**). Not only does the self-assembling peptide allow spontaneous chondrogenesis, but it also promotes, in certain conditions, a unique *in vitro* cellular self-organization that resembles early embryonic stages (**Figure 2 C**). Furthermore, similarly to what has been observed in animal development, the progression of this morphogenesis is dependent on proliferation as evidenced by the effects of Staurosporine on the system contraction (**Figure 2 E** and **F**). It was also noticed that less initial concentration of MEFs ($5 \cdot 10^5$ cells/ml) within the RAD16-I slowed three times the contraction of the cell mass and failed to render bilaterality (results not shown). These results indicate that proliferation seems to have a close relationship with the spontaneous chondrogenic differentiation of MEFs, similar to the relationship between the embryonic mesenchymal condensation and chondrogenesis within the pre-skeletal tissue. Altogether, as a consequence of the biomechanical and non-instructive properties of RAD16-I scaffolds, we can speculate that the contraction produced during the morphogenetic process could reproduce a mesenchymal condensation phenomenon that explains the default chondrogenic pathway. Hence, the localized natural chondrogenic induction could be the result of mesenchymal condensation and differentiation under the control of an organized process that directs tissue-like patterning. This is a significant difference from the mesenchymal potential of fibroblasts in high-density micromass cultures, because in these cultures, although fibroblasts reproduce mesenchymal condensation and show default chondrogenic commitment, no self-organization is observed [76, 77].

We have also showed, by immunohistochemistry and *in situ* hybridization, that the expression of Brachyury, and its localization in this system, is highly related to the formation of the 3D-bilaterality (**Figure 9 A** and **B**). First, Brachyury is expressed in a mid-line zone of the structure, to further spread at both sides of the central axis, suggesting that mesodermal