Published in final edited form as:

J Vasc Res. 2012 ; 49(5): 441–446. doi:10.1159/000339568.

Focal High Cell Density Generates a Gradient of Patterns in Self-Organizing Vascular Mesenchymal Cells

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Abstract

In embryogenesis, structural patterns, such as vascular branching, may form via a reaction-diffusion mechanism in which activator and inhibitor morphogens guide cells into periodic aggregates. We previously found that vascular mesenchymal cells (VMC) spontaneously aggregate into nodular structures and that morphogen pairs regulate the aggregation into patterns of spots and stripes. To test the effect of a focal change in activator morphogen on VMC pattern formation, we created a focal zone of high cell density by plating a second layer VMC within a cloning ring over a confluent monolayer. After 24 hours, the ring was removed, and pattern formation monitored by phase-contrast microscopy. At days 2–8, the patterns progressed from uniform distributions to swirl, labyrinthine, and spot patterns. Within the focal high-density zone and a narrow halo zone, cells aggregated into spot patterns; in the outermost zone of the plate, cells formed a labyrinthine pattern. Area occupied by aggregates was significantly greater in the outermost zone than in the HDZ or halo. The rate of pattern progression within the HDZ increased as a function of its plating density. Thus, focal differences in cell density may drive pattern formation gradients in tissue architecture, such as vascular branching.

Keywords

vascular; pattern; calcification; self-organization

Introduction

During embryonic development, the formation of some tissues, such as branching vessels, nerves, bones, and hair follicles, involves pattern formation at a microscopic and/or macroscopic level. During development, periodic structures may create a framework for macroscopic patterns that form complex biological structures [1,2]. The underlying mechanisms responsible for the migration and selforganization of cells into macroscopic structures remain under intense investigation, especially as it translates to clinical tissue engineering in the treatment of degenerative disorders. Previous investigators have shown that vascular cells, including microvascular pericytes and cardiac valvular cells, can migrate and contract in an organized manner to form nodules *in vitro* [3,4].

Turing's reaction-diffusion concept [5] may explain many forms of biological pattern formation such as stripe and spot patterns, based on a relatively simple system of activator and inhibitor molecules, termed morphogens, interacting under the right conditions of

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autocatalysis and relative diffusivity, and creating periodic structures. Reaction-diffusion phenomena may account for variation in size, number, and distribution of periodic patterns in feather primordia, hair follicles, and fur patterns in animals [6,7,8,9,10]. Importantly, in general, this phenomenon relies on the inhibitor morphogen having a much higher diffusion coefficient than the activator.

We previously showed *in vitro* pattern formation by vascular mesenchymal cells (VMC), a subpopulation of smooth muscle cells previously known as calcifying vascular cells, which also have multilineage potential based on RT-PCR, Western, and flow cytometric analyses [11]. These cells form swirls (areas of local alignment, without aggregation), stripes (multicellular, raised, elongated aggregates), spots (multicellular, raised, circular aggregates), or labyrinthine patterns (stripes in a maze-like pattern) [12].

We previously found that this progression is driven by reaction-diffusion governed by two morphogens: an activator, bone morphogenetic protein-2 (BMP-2), and its inhibitor, matrix gamma-carboxyglutamic acid protein (MGP). From promoter-reporter construct analysis, BMP-2 expression is regulated in an autocatalytic manner [13]. Computer simulation of the reaction-diffusion mathematical model predicted labyrinthine patterns under control conditions, spot patterns with exogenous MGP, and increased frequency of the pattern's periodicity following treatment with the MGP inhibitor, warfarin [12]. *In vitro* experiments confirmed these predictions. A more complete solution-space of the system of partial differential equations, corresponding to a wide spectrum of patterns, was characterized by Yochelis et al [14].

Danino and colleagues [15] used a pseudospectral technique and fast Fourier transform methods to expand this model and computer simulation to the three-dimensional level. Their results predicted a range of patterns including evenly-spaced spheres, bands, or tubes, as a function of the values of coefficients assigned to the activator saturation and activator degradation terms in the equations.

We reasoned that, if a point source of activator were created within a culture, it may produce a radial gradient of patterns with increasing sparseness distributed as the activator/inhibitor ratio decreases beyond the point source. Since a non-biological carrier of BMP-2, such as a sponge or drip mechanism, would lack the autocatalytic effects of cell-derived BMP-2, in this study, we chose to create a local cellular source of additional BMP-2 (activator) by plating additional VMCs in a small, high-density zone (HDZ) in each culture. This intervention resulted in a progressive change in pattern within the HDZ and a spectrum of patterns extending radially from it.

Methods

Primary cultures of bovine aortic mesenchymal cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Mediatech, Manassas, VA) with 15% fetal bovine serum and used at passages 16–17. Cells were initially plated in 6-well plates (BD Falcon 6-well Multiwell Plate, Becton-Dickinson BD Labware, Franklin Lakes, NJ) at 200×10^3 cells/well and cultured until confluency. After 2 days, when cells were confluent, a second set of cells was plated (20, 40, 60, 80, 160 and 320×10^3 cells/ring) within an ~1-cm cloning ring. (Although the ring area is approximately 0.8 cm^2 , we approximated it as 1 cm^2 in reporting values of plating density, for simplicity and because the cells spread slightly after removal of the ring). A second, control cloning ring without additional cells was placed in and removed from each well. Cloning rings were removed after another 2 days, and pattern formation was monitored daily by digital acquisition of phase-contrast microscopic images (Olympus CKX41; Olympus, Center Valley, PA). After 8–10 days, cells were fixed with 4 % (w/v)

formaldehyde in phosphate-buffered saline and stained with toluidine blue, pH 6.4. In BMP-2 inhibition experiments, 300ng/ml of mouse noggin (Recombinant Mouse Noggin 1967-NG-025; R&D Systems, Minneapolis, MN) were added on day 2 and experimental wells were retreated with 300ng/ml of mouse noggin every 2 days.

Digital phase-contrast microscopic images were analyzed to determine the percent area occupied by cellular aggregates, such as spots or stripes. We selected for the spots or stripes and used area analysis in Photoshop CS4 Extended (Adobe Systems; Mountain View, CA). Cell density of spots was analyzed using densitometry gel blot analysis in Image J (Image J 1.45s; NIH, Bethesda, MD). Values are expressed as mean $\pm$ SD. Comparisons of percent area were performed using Student's *t*-test. The criterion for significance was $p < 0.05$.

Results

Effect of a high-density zone on pattern formation

The presence of the high-density zone (HDZ) produced a gradient of patterns by day 10. Within the HDZ, cells formed a labyrinthine pattern; at the outer edges of the dish, they formed a stripe pattern; and, in between, a spot pattern (Fig. 1). No such pattern gradient was seen in the control dishes where a cloning ring was placed but not plated with any additional cells, suggesting that the observed changes are not attributable to cell injury from manipulating the cloning ring.

Effect of cell density on pattern formation

To test the effects of cell density in the HDZ on pattern gradient, we plated of a range of cell densities (from 20×10^3 to 320×10^3 /ring) in HDZ generated on confluent, day-3 monolayers. At the lowest HDZ density (20×10^3 cells), no discrete pattern formation was observed (data not shown). At the next lowest HDZ density, the culture formed swirls; at the next higher density, stripes; the next higher density, labyrinthine pattern; and at the highest density, spots (Fig. 2). Higher HDZ densities accelerated the time-course of pattern progression as described below.

Time course of pattern progression

The patterns also changed over time, in an HDZ-density dependent manner. On day 8, the HDZ appeared to settle into a stable spot pattern independently of HDZ density. However, the rate at which cells reached this steady-state was dependent on HDZ density. Cultures with lower HDZ densities exhibited uniform cellular distribution or premature swirl morphology for days 1–3, progressing to stripes, labyrinths, and spots through day 8 (Fig. 3). At HDZ densities of 160 – 320×10^3 cells/cm², spots formed within 24 hours of HDZ plating. In contrast, at HDZ densities of 20 – 40×10^3 cells/cm², spot patterns did not appear until 3 days after plating the HDZ layer.

Cellular alignment and orientation

High magnification images of the edge of the HDZ show elongation, alignment, and orientation of cells perpendicular to the surface of a raised aggregate (Fig. 4). In some areas, this self-organized ridge condensed into nodules.

Gradient of sparseness

When the HDZ was centered within the well, a gradient of pattern sparseness was evident. The HDZ and a halo zone surrounding it had a sparser pattern of aggregates than the remainder of the dish (Fig. 5). Inside the HDZ, the percent area occupied by spot-shaped aggregates was $18 \pm 3\%$. In a halo zone immediately surrounding the HDZ, the percent area

occupied by these aggregates was similar, $22 \pm 7\%$. In the remainder of the dish, where the cells aggregated into a labyrinthine pattern similar to that of control cultures, the percent area occupied by aggregates was significantly greater, $48 \pm 7\%$. Thus, the outer zone had nearly 3-fold more area occupied by aggregates than did the HDZ ($p < 0.001$) and 2-fold more area occupied by aggregates than the halo zone ($p < 0.001$) (Fig. 5).

Edge effects

In cultures with an HDZ plated at 60×10^3 cells/cm², nodules arose primarily at the edge of the HDZ (Fig. 6A). When the HDZ was plated at 80×10^3 cells/cm², nodules arose uniformly throughout the HDZ (Fig. 6B). To exclude the possibility that the temporary placement of the cloning ring produced these effects by injuring the culture, we performed the primary and secondary plating in reverse order ("reverse" plating); the HDZ was plated first, then the cloning ring was removed on day 2 and the entire well plated second. The control experiments showed no effect of the reverse plating method (Fig. 6C).

Effect of BMP-2 inhibition

To test whether morphogen activity has a role in the pattern gradient, we manipulated BMP-2 activity by adding its known inhibitor, noggin, at a concentration known to inhibit BMP to a degree equal to that of siRNA [16]. Noggin (300 ng/ml) was added on day 2, and for an additional 6 days. After 6 days, the percent area occupied by aggregates in control wells was 2-fold greater than noggin-treated wells (44 ± 10 vs. 25 ± 0.6 ; $p = 0.04$), suggesting a delay in pattern and nodule maturation with BMP-2 inhibition.

Discussion

It was previously shown that vascular mesenchymal cells self-organize into macroscopic aggregates in distinct, periodic patterns in culture. The present findings indicate that, when these cells are plated with a focal area of high cell density, multiple periodic aggregates arise with sparseness increasing with proximity to the high-density zone. The pattern gradient formed most rapidly when cells were plated at highest density in the high-density zone. Based on our previously described, reaction-diffusion mathematical model, we speculate that the sparse pattern within the high-density zone is due to high activity levels of the morphogen, BMP-2, and its inhibitor, matrix GLA protein. At distances beyond the high-density zone, the low activity levels of both BMP-2 and MGP result in a dense pattern.

Other investigators have also found evidence for reaction-diffusion phenomena in biological pattern formation, such as hair follicle patterns driven by the morphogen Wnt and its inhibitor, DKK [17]. Notably, Wnt signaling has inhibitory crosstalk with BMP-2 [18]. In their 3-dimensional computer simulations, Danino and colleagues [15] found that an increase in levels of the activator morphogen (produced by assigning a low saturation constant to BMP-2 autocatalysis), would yield a spot pattern. This is consistent with our finding of a spot pattern within the focal HDZ, though generated by a different mechanism.

We speculate that the high cell density zone in these experiments creates a focal increase in BMP-2 activity (activator morphogen), as well as a halo zone, where activity of its inhibitor, MGP, predominates. Since BMP-2 activity is expected to saturate more quickly than its inhibitor [13,19], a radial gradient of morphogen activities and hence a local gradient of macroscopic cell pattern may result. Our experiments confirm that the HDZ appears to create a radial gradient of patterns. Within the cloning ring, a pattern of stripes progressed over time to spots, with more rapid progression at higher levels of plating density. Immediately outside the cloning ring, a halo of sparse periodic nodules arose. At a further distance, a dense labyrinthine pattern formed. This gradient (Fig. 5) may be due to rapid

diffusion of the small inhibitor protein, resulting in a high ratio of activator-to-inhibitor activity inside the HDZ, a low ratio of activator-to-inhibitor activity in the halo zone, and a low level of both beyond the halo. Our finding that noggin treatment modifies the pattern supports a role of this activator-inhibitor pair and reaction-diffusion in the effects of formation.

These findings may explain, in part, how pattern gradients and tissue architecture form during embryonic development. In addition, such principles may be applied to tissue engineering to produce more naturally patterned architectures in tissues. Due to the limited supply of donor organs and subsequent problems with immunosuppression, tissue engineering is a critical therapeutic approach to degenerative disease. It would be highly desirable to engineer tissue from a patient's own stem cells. However, tissues and organs are not simply collections of cells. Organ function requires architecture created by nodular, labyrinthine, stripe, and swirl patterns/zones. It is currently not clear how cells can distinguish and emerge without a global guidance. Here, our finding of cells' inherent capacity to organize themselves provides a fundamental answer to this question.

Acknowledgments

This work was supported, in part, by NIH grants HL081202 (LD) and DK081346 (YT), as well as NSF grant 1025073 (AG).

References

1. Murray JD. A prepattern formation mechanism for animal coat markings. *J Theor Biol.* 1981; 88:161–199.
2. Shoji H, Iwasa Y, Kondo S. Stripes, spots, or reversed spots in two-dimensional Turing systems. *J Theor Biol.* 2003; 224:339–350. [PubMed: 12941592]
3. Doherty MJ, Ashton BA, Walsh S, Beresford JN, Grant ME, Canfield AE. Vascular pericytes express osteogenic potential in vitro and in vivo. *J Bone Miner Res.* 1998; 13:828–838. [PubMed: 9610747]
4. Chen JH, Yip CY, Sone ED, Simmons CA. Identification and characterization of aortic valve mesenchymal progenitor cells with robust osteogenic calcification potential. *Am J Pathol.* 2009; 174:1109–1119. [PubMed: 19218344]
5. Turing AM. The chemical basis of morphogenesis. *Philos Trans R Soc.* 1952; B237:37–72.
6. Nagorcka BN, Mooney JR. The role of a reaction-diffusion system in the formation of hair fibres. *J Theor Biol.* 1982; 98:575–607. [PubMed: 7154690]
7. Nagorcka BN. Evidence for a reaction-diffusion system as a mechanism controlling mammalian hair growth. *Biosystems.* 1983; 16:323–332. [PubMed: 6673782]
8. Nagorcka BN, Mooney JR. The role of a reaction-diffusion system in the initiation of primary hair follicles. *J Theor Biol.* 1985; 114:243–272. [PubMed: 4033155]
9. Lin CM, Jiang TX, Baker RE, Maini PK, Widelitz RB, Chuong CM. Spots and stripes: pleomorphic patterning of stem cells via p-ERK-dependent cell chemotaxis shown by feather morphogenesis and mathematical simulation. *Dev Biol.* 2009; 334:369–382. [PubMed: 19647731]
10. Lin CM, Jiang TX, Baker RE, Maini PK, Widelitz RB, Chuong CM. Spots and stripes: pleomorphic patterning of stem cells via p-ERK-dependent cell chemotaxis shown by feather morphogenesis and mathematical simulation. *Dev Biol.* 2009; 334:369–382. [PubMed: 19647731]
11. Tintut Y, Alfonso Z, Saini T, Radcliff K, Watson K, Bostrom K, Demer LL. Multilineage potential of cells from the artery wall. *Circulation.* 2003; 108:2505–2510. [PubMed: 14581408]
12. Garfinkel A, Tintut Y, Petrusek D, Boström K, Demer LL. Pattern formation by vascular mesenchymal cells. *Proc Natl Acad Sci.* 2004; 101:9247–9250. [PubMed: 15197273]
13. Ghosh-Choudhury N, Choudhury GG, Harris MA, Wozney J, Mundy GR, Abboud SL, Harris SE. Autoregulation of mouse BMP-2 gene transcription is directed by the proximal promoter element. *Biochem Biophys Res Commun.* 2001; 286:101–108. [PubMed: 11485314]

14. Yochelis A, Tintut Y, Demer LL, Garfinkel A. The formation of labyrinths, spots and stripe patterns in a biochemical approach to cardiovascular calcification. *New J Phys*.
15. Danino T, Volfson D, Bhatia SN, Tsimring L, Hasty J. In-silico patterning of vascular mesenchymal cells in three dimensions. *PloS One*. 2011; 6 e20182.
16. Bostrom K, Jumabay M, Matveyenko A, Nicholas SB, Yao Y. Activation of Vascular Bone Morphogenic Protein Signaling in Diabetes Mellitus. *Circ Res*. 2011; 108:446–457. [PubMed: 21193740]
17. Sick S, Reinker S, Timmer J, Schlake T. WNT and DKK determine hair follicle spacing through a reaction-diffusion mechanism. *Science*. 2006; 314:1447–1450. [PubMed: 17082421]
18. Ille F, Atanasoski S, Falk S, Ittner LM, Marki D, Buchmann-Moller S, Wurdak H, Suter U, Taketo M, Sommer L. Wnt/BMP signal integration regulates the balance between proliferation and differentiation of neuroepithelial cells in the dorsal spinal cord. *Dev Biol*. 2007; 304:394–408. [PubMed: 17292876]
19. Zebboudj AF, Shin V, Boström K. Matrix GLA protein and BMP-2 regulate osteoinduction in calcifying vascular cells. *J Cell Biochem*. 2003; 90:756–765. [PubMed: 14587031]

Figure 1.

Light microscopic images of toluidine blue-stained vascular mesenchymal cell cultures in 6-well plates at 10 days. The left dashed circle indicates where the cloning ring was applied temporarily to add the second layer of cells (400×10^3 cells/cm²) to create a high-density zone (HDZ). The right circle indicates where a control cloning ring was applied without additional cells. Scale bar = 1cm.

Figure 2.

Phase contrast microscopy of VMC cultures grown in 6-well plates, where cloning rings were used to create HDZs with densities ranging from 20×10^3 to 320×10^3 cells/cm². Images were taken within the HDZ on day 3. Patterns progressed from swirls to labyrinthine and spot patterns with increasing cell density. Dashed lines indicate swirls, arrows indicate spots, chevrons indicate edges of stripes, and curved solid lines outline labyrinths. Scale bar = 0.1 mm. Magnification 40X.

Figure 3.

Phase contrast microscopy at days 2 to 8 of VMC cultures grown in 6-well plates, where cloning rings were used to create high-density zones of 20×10^3 cells/cm². Images were taken within the high-density zone. Patterns progressed from swirls to labyrinthine and spot patterns with increasing cell density. Scale bar = 0.1 mm. Magnification 40X.

Figure 4.

High magnification phase contrast microscopy of the high-density zone at 40X (A), 100X (B), 100X (C), and 400X (D) showing elongation and alignment of cells perpendicular to the edges of the aggregates. Arrows in 4B indicate individual elongated and aligned cells.

Arrows in 4C and 4D indicate the orientation of the elongated cells. Lines in 4D indicate the axis of the edge of the nodule. Scale bars are approximately 0.1 mm (A), 0.04 mm (B), 0.04 mm (C), 0.01 mm (D).

Figure 5.

Light scan (1X) of toluidine blue-stained VMC cultures in 6-well plates at 8 days. The dashed lines indicate the edges of the HDZ, where the secondary VMC layer was plated at 160×10^3 cells/cm². The solid line indicates the border between the sparse pattern in the halo area and the denser labyrinthine and stripe patterns. Scale bar = 1cm.

Figure 6.

Light microscopic images of toluidine blue-stained VMC in 6-well plates at 3 days, where cloning rings were used to create high-density zones ranging from 60×10^3 to 80×10^3 cells/cm². The dashed circle shows where a second layer of VMC was applied to a uniformly distributed layer of VMC.

A. At 60×10^3 cells/cm², a labyrinth pattern formed with nodules at the edge of the HDZ. Scale bar = 2mm.

B. At 80×10^3 cells/cm², nodules are evenly distributed within the HDZ. Scale bar = 2mm.

C. In the reverse plating method, the HDZ was plated first, and the entire well plated after removal of the cloning ring, to exclude possible injury from manipulation of the cloning ring. Scale bar = 2mm.