JACOBS PUBLISHERS

OPEN ACCESS

Jacobs Journal of Regenerative Medicine

Review article

Simple Tools for Structuring Embryonic Rudiments

Beloussov LV*, Kremnyov SV, Luchinskaia NN

Laboratory of Developmental Biophysics, Department of Embryology, Faculty of Biology, Moscow State University 119991 Moscow

**Corresponding author: Dr. Beloussov LV, Laboratory of Developmental Biophysics, Department of Embryology, Faculty of Biology, Moscow State University 119991 Moscow, Email: morphogenesis@yandex.ru*

Received: 08-24-2015

Accepted: 11-26-2015

Published: 12-22-2015

Abstract

In this paper we review experimental approaches used in our research group for deforming embryonic tissues in amphibian embryos by relaxing pre-existed tensions, stretching samples in given directions or bending them. In these experiments, owing to the active tissue reactions to changes in mechanical stresses, they change their shapes in predictable ways. In some cases the changes in geometry dictate reconstruction of cell differentiation patterns. We suggest that these results may orient bioengineers in elaborating new technologies permitting to endow artificial organs by required shapes.

Keywords: Mechanical stresses; Xenopus embryos ; Cell movements; Tissue deformations ; Curvatures

Introduction

It is almost trivial to remind that the samples which the regenerative medicals are striving to construct should not be amorphous. To give them a properly controlled shape and internal structure instead of being satisfied by what is arisen spontaneously in cultivated pieces of tissue is a challenging task. In this essay we describe some simple approaches used in our lab for providing pieces of embryonic tissues by a desired spatial structure in the hope that manipulations which we used for apprehending the fundamental laws of morphogenesis may be already now of some applicatory value.

We use the term "structuring" in a broad sense as embracing both creation of new geometric shapes and more refined changes in tissue structure, such as formation of columnar cells domains (placodes) out of homogeneous epithelial sheets, creation of smooth borders within dense cell masses, collective cell migrations in desired direction(s), loss and acquiring of epithelial structure, etc. (Figure 1). As a result of several decades studies (starting point: [1]) we concluded that all of these processes are controlled by endogenous mechanical stresses (MS), mostly tensile ones, so that by modulating MS at the proper stages of development we can affect any one of them. As indicated by increasing number of evidences, MS are crucial for development of all Metazoans, from Cnidaria to Amniotes. In particular, the abolishment of tensions on the surface of yolk sac hampers the development of chicken embryos from the very start of incubation [2].

For approaching rationally to bioengineering aspects of shape formation it is important to apprehend that it is based upon the active mechano-chemical reactions of embryonic tissues to MS, the latter being in normal development generated mainly by immediately preceded morphogenetic movements. Accordingly, a chain of feedbacks should be established within developing embryos between MS patterns and successively arisen shapes [3-6]. In this context, what can be done by a bioengineer is to modify MS patterns in the hope to get from embryonic tissues the expected morphological responses. Although the system of MS - shapes feedbacks is still far from being elucidated in all the details, some of its properties are already more or less clear. We in our group employ for this purpose a so called hyperrestoration (HR) model which claims that embryonic tissue responds to any (normal or artificial) MS change by generating forces directed towards the restoration of initial MS value but overshooting it to another side. Similarly, whenever such MS changes are unevenly distributed or are anistropic, then the responses will be directed towards reducing with an overshoot

whichever deviations were greater [3,7]. The examples to be presented below will illustrate the applications of HR model.

It is of a primary importance for bioengineering purposes to know to what extent the cell differentiation patterns are linked with morphogenesis in its strict sense, that is, embracing nothing more than embryonic geometry and topology. In other words, to what extent (if any) cell differentiation is mechanodependent? A number of recent data [8-11] shows that in spite of a certain autonomy of the both processes, such a dependence may be greater than it could be suggested beforehand. One of below presented experimental models will be the illustration.

Actually we have used in our experiments no more than the following three kinds of mechanical procedures:

- Relaxation of tensions;
- Imposing tensions in abnormal directions;
- Artificial bending of tissue pieces.

Results of tensions relaxation: multiplication of anlagens, formation of abnormal protuberances, enlarging of epithelial placodes.

Tensions in amphibian embryonic tissues can be relaxed by different ways. A simple method to do this at the blastula stage is to temporally reduce turgor pressure in blastocoel which normally stretches so called blastocoel roof, later giving rise to ectodermal layer [12]. An effective way to do the same at the gastrula stage (when blastocoel is already largely reduced) is to insert within a vegetal embryo hemisphere a sector of homologous tissue (endodermal "wedge" – Figure 1A, E), thus slightly compressing embryonic ectoderm [13]. For relaxing tensions within a small piece of tissue, a "remove – put it back" procedure can be performed [14]: a piece of tissue (including ectoderm) should be extirpated from embryo and in about a minute returned back to the same position (Figure 1 I); during this brief time period the piece is contracted and wound gap enlarged, so that the returned piece cannot establish lateral contacts with surrounding tissues which is necessary for restoring the initial tensions.

In these entire cases one should take into mind that if a tissue remains alive, a real relaxation of tensions will last no more than for few minutes: more prolonged loss of tensions switches on a program of cell apoptosis [15]. Estimations of tensions based upon cells geometry show indeed that the restoration is fast enough and goes with overlapping [13]. The matter is however that practically in no cases the initial (normal) tensile pattern is spontaneously restored: as a rule the regular global patterns embracing the entire embryonic body are replaced by more local and irregular ones. As a result, the main outcome of any of the above described procedures is a loss of a long-range morphological order while more local processes (including cell differentiation) remain almost non-affected (Figure 1D, F, G, J). This is also a tool to make tissue protuberances (Figure 1 B, C) and to multiply homologous rudiments, in the given case neural tubes (Figure 1G).

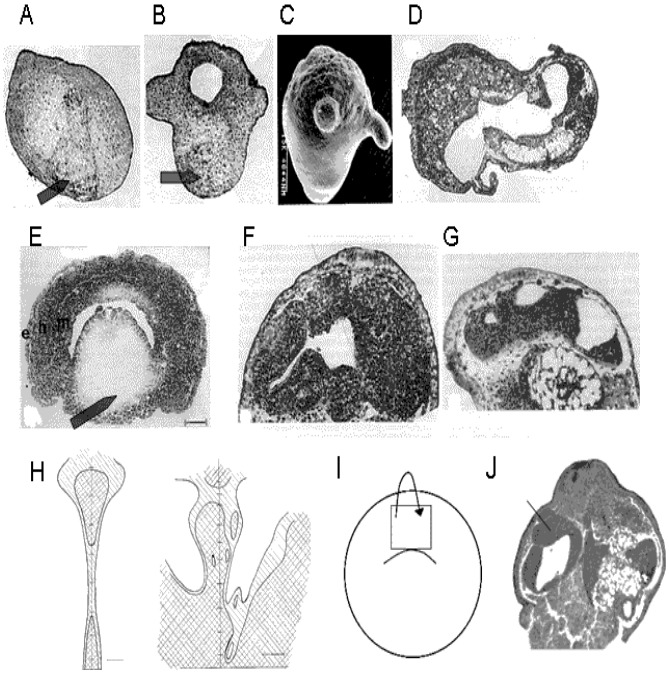

Figure 1. Relaxation-induced effects in *Xenopus* embryos. A: a wedge of a ventral tissue (pointer) is inserted in a blastula stage embryo. B-D: operated embryos, 5, 24 and 48 h after insertion, correspondingly. Note abnormal protuberances in B, C and a complete loss of order in D. E: a similar wedge (pointer) inserted in the early gastrula stage embryo (E) produces abnormal transversal extension (F) or multiplication (G) of neural tubes. H: 2-dimensional maps of cells height/width (H/W) relations in the dorsal ectoderm of an intact (left) and relaxed (right) neurula stage embryo. Cross-hatched area is that filled by most columnar cells. I: a scheme of a "remove – put it back" procedure. J: a resulted chaotic arrangement of axial organs in the operated region. A-C: from Beloussov et al., [12]; D-H from Beloussov et al., [3]; I, J: from Kornikova et al., [14].

Another typical consequence of relaxation is the enlargement of columnar cell areas (usually called placodes) at the expense of flat epithelia (Figure 1 H). So far as formation of placodes is usually connected with cell differentiation (neuralization, development of sensory cells) these transformations may be of a particular value for regenerative medicine.

Decrease of tensions induce epithelial cells to make first steps of a so-called epithelio-mesenchymal transition (EMT), that is, endocytotic engulfment of the apical membrane and acquirement of bottle-like shape [16]. This provides a simple experimental model for studying EMT which is known to be one of the crucial steps of oncogenesis.

Consequences of controlled tissue stretching: triggering convergence-extension cell movements and molding various axi-symmetric shapes.

We use two methods for stretching embryonic tissues. By the first one, tissue explants are placed onto specially pretreated pieces of latex films to which they are adhered by their naked surface (opposite to the outer one), consisting in Anuran amphibian embryos of so called inner ectoderm cells, loosely connected with each other. The films are mounted in the wheel-supplied devices permitting to stretch films together with attached explants in a desired temporal regime [17]. In spite of these advantages, this technique is rather capricious and is hardly suitable for mass experiments. For the latter purposes it is easier to stretch explants in a step-wise manner by two pairs of needles stick into agarose substrate [18]. Another advantage of this method is that the stretching can be applied to double explants (sandwiches) completely isolated from external environment, rather than to naked explants, as in the previous case.

In the both cases the main result was stimulation of so called convergence-extension cell movements which normally play a leading role in formation of so called axial organs (neural tube, notochord, somites) in all the Vertebrate embryos. For coming into more details, we have to become familiar with a regional structure of amphibians embryonic bodies. At the gastrula stage they consist of two parts, called suprablastoporal and blastocoel roof areas (SBA and BRA correspondingly). During normal development the convergent-extension movements are going on within SBA only, just giving rise to the axial organs elongated in antero-posterior (AP) embryo direction. By stretching SBA explant perpendicularly to AP axis (that is, transversely) it is possible to reorient the axial organs (and in the first turn the notochord) in this direction. By making a similar experiment with BRA explant the convergent-extension cell movements (never presented normally in this area) can be triggered as well, but in no cases this will lead to formation of something similar to the axial organs.

Nevertheless, the resulted shapes of stretched BRA explants are rather peculiar and various, differing from what may be expected in the forcibly elongated samples: one can see among them dumb-bell and horse shoe-shaped configurations (Figure 2D-F). Also, in many cases the lateral surfaces (to which the stretching force is directly applied) become, contrary to expectations, undulated. All of this indicates that, as predicted by HR model, in response to outside stretching the internal pressure forces oriented just in stretch direction are arisen within a sample. This permits, by varying a total amount and periodicity of stretching to obtain a large repertoire of axi-symmetric shapes.

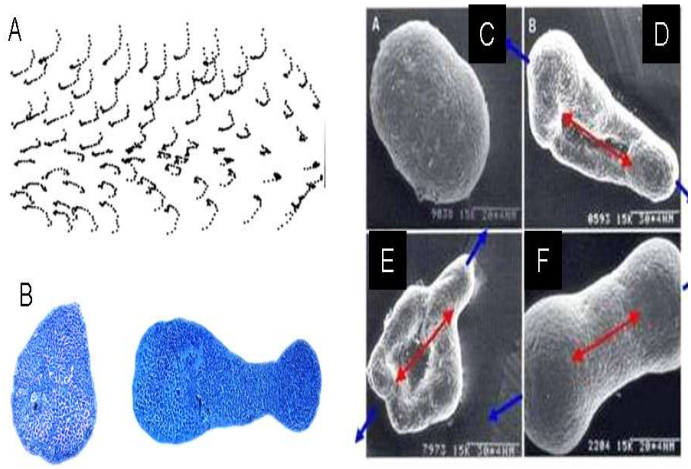

Figure 2. Effects of stretching embryonic tissues. A: a typical vector field of cells convergence-extension triggered by stationary tissue stretching in horizontal direction. Redrawn from time-lapse film 30 min duration. B (from left to right): transformation of horizontally stretched explant of SBA tissue into a dumb-bell notochord rudiment, elongated perpendicularly to its normal direction. C: a control (non-stretched) VLA explant. D-E: various shaping of VLA explants stretched on latex films. A: from Troshina et al., [18]. B: from Mansurov, [19]. C-F: courtesy of N.N. Luchinskaia.

Artificially imposed curvature can be actively enhanced; within a competent tissue this may affect cell differentiation patterns.

If preparing a sandwich from BRA tissue and try to fasten it in the bending state (by pulling into a groove) in the first dozens of seconds it resists bending as an elastic body, attempting to straighten back again. Within few minutes however it not only takes an imposed shape but tries to reinforce it actively by increasing the bending curvature due to contraction of the apical cell surfaces [20] (Figure 3). Such a passage from passive to active bending seems to play an important role in making embryonic shapes and may be hence recommended for bioengineering purposes. Moreover, while the bending of a BRA-produced tissue sample does not lead to any changes in cell differentiation patterns, this is not the case for SBA samples [21]. Just a slight bending of SBA sandwich leads not only to extensive curvature increase of a slight ingression (Figure 4 cf A and B) but to a curvature-dependent differentiation into neural and muscle tissue: the neural cells (dark blue) are differentiated as a compact cluster in the concave area of a double explant while muscle cells (red) are formed closer to the convex area (Figure 4C, D). So far as sandwich' layers becoming due to bending concave and convex were initially equivalent, the obtained pattern shows that within SBA cell differentiation can be controlled by artificially imposed geometry.

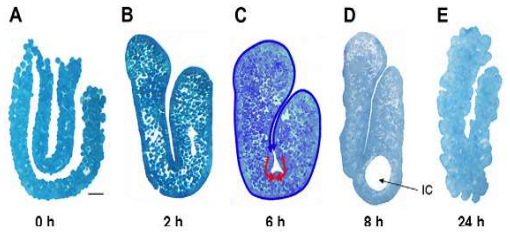

Figure 3A-E. Stabilization and increase of artificially imposed curvature in tissue sandwiches prepared from BRA. Times after operation are shown. Red converged arrows in (C) display active contractile forces while blue lines depict stretched surfaces which later on (E) create numerous undulations (see below for comments). IC: inflated cavities in the inner parts of tissue sandwiches [20].

Relations between local curvatures and cells rearrangements: bioengineering perspectives.

Under enough prolonged incubation the double BRA explants acquire, due to ions and water transport, the osmotically pressurized internal cavity and are thus transformed into vesicles. Their shapes rarely become precisely spherical: usually the regions of a high curvature are alternated with more flattened ones. As claimed by the Laplace law, the local tensions in the walls of pressurized cavities are reversely proportional to the curvatures: the more a given piece of the wall is curved, the less it is tensed, and vice versa. As it follows from HR model, the local MS should go towards equalization with an overlap what means that the tensions in the mostly curved regions should increase while those in less curved ones to go down. This means that the surface of the mostly curved regions is to be diminished while that of the less curved to be increased. To achieve these results, the cells of the mostly curved regions should either contract transversely (that is, become more columnar) or emigrate out of a cell sheet, that is, undergo EMT; at the same time, the mostly stretched flattened areas have to relax the surface becoming more thin; in the case of extensive overlapping this reaction can lead to undulated configuration of the surface.

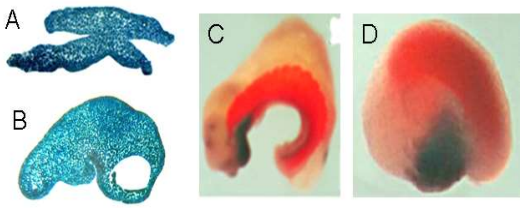

Figure 4. Correlations between artificially imposed shape and cell differentiation patterns in double explants of SBA tissue. A: slightly bent double explant before a complete sticking of its layers. B: a similar explant 3 h later acquiring a bent shape. Note formation of a narrow curved ingression on the concave side. C, D: in situ-hybridized 24h explants. Neural tissue (dark blue) is located on the concave side of explant, while muscle tissue (mesodermal somites) (red) form a band shifted towards convex side [21].

Just these results are observed both in the normal development and in BRA sandwiches (Figure 5A-C). In tune with these expectations, undulations are formed along the mostly stretched areas of the forcibly bent sandwiches (see Figure 3E). The curvature-dependent cell rearrangements should be taken in consideration by the bioengineers interested to control refined details of cells shaping and rearrangements.

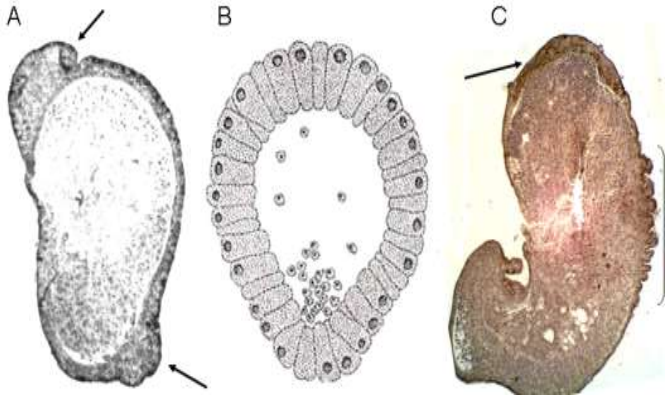

Figure 5. Relations between curvatures and cells rearrangements. A, C, arrows: formation of columnar cell domains in the areas of maximal curvature. B: immigration of cells from the maximal curvature area. Bracket in C denotes undulations on the convex (mostly stretched) area. A: BRA sandwich; B: normally developing sponge larva. C: Xenopus embryos, artificially arrested at the early gastrula stage. From Beloussov [7].

Making tubes out of rolls

The rolls (toroids) consisting of a dense inflated tissue are among widely spread embryonic rudiments: so called lips of circular blastopores are the best known examples. A universal property of inflated toroid bodies with stretchable envelopes is inequality of tensions in the different directions. As known from mechanics, the transversal (meridian, or circular) surface tensions in such bodies are twice as great as the equatorial ones. If applying to these bodies the above described tendency towards smoothing out with an overshoot the tensions inequalities, one should expect the meridian tensions (being initially the greatest ones) to be firstly diminished and then replaced by similarly oriented pressure stresses (as in examples illustrated by Figure 2D-F). This can be done only by cells convergence towards meridians (Figure 6A, B, converging arrows) which provides equatorial contraction and hence (due to tissue incompressibility) elongation of a toroid body in meridian direction. As a result, it will be transformed into a tube. Such processes are ubiquitous for normal development (formation of intestine out of a blastoporal lip material is an example) and can be easily reproduced experimentally (Figure 6C). For initiating tube formation, it is enough to prepare a ring of tissue consisting of cells able to convergent intercalation between each other.

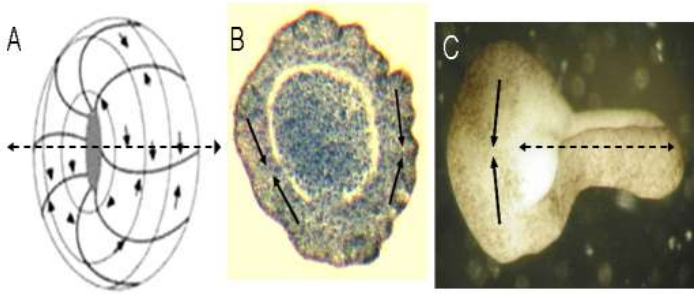

Figure 6. Making tubes out of rolls. A: A toroid tissue body. Arrows depict cell convergent movements directed towards meridians for releasing maximal tensions and exchanging them by meridionally oriented pressure providing axial elongation of a body (dashed bidirectional arrow). B: a cross-section of a blastopore-adjacent zone in Xenopus embryo illustrating the directions of cells convergence. The folds indicate equatorial shrinkage. C: a tubular formation spontaneously arisen from the tissue ring isolated from Xenopus early gastrula embryonic tissue.

Attempts to quantify the forces deforming embryonic tissues

Several recent attempts to measure deforming forces and Young modulus of embryonic tissues [22-24] led to results which differ from each other in an order or more. By our suggestion, these discrepancies are due to the lack of a common agreement in defining tissue areas to which external forces are applied and to mixing the latter's direct effects with those of the active tissue responses. For example, in our evaluations of Young modulus values in the early gastrula *Xenopus* embryos with the use of sucking experiments [23] we took as denominator the section area of a sole cell layer which could resist the deforming force (this is so called epiectoderm)
while Von Dassow and Davidson [22] used in this role the entire section area of embryonic tissue consisting mostly of non-supportive endoderm. In addition, we took for measurements only the force values detected in few minutes after the start of deformation (when the active reactions enhancing the deformation have not still developed) while the mentioned authors employed more prolonged measurements. As a result, we got Young modulus value E = 4,1 ± 0,6 kPa (this is within the range typical of soft biological materials: [25]) while Von Dassow and Davidson data [22] were in about two orders smaller. In general, the contribution of the active responses having their own regional and stage-specific dynamics in no way can be neglected and to a large extent depreciates formally correct measurements. For example, active enhancement of externally imposed deformation typical for gastrula stage embryos is exchanged at the advanced stages by a strong oppositely directed reaction giving the impression of enormous increase of Young modulus [26]. Taking into mind these reservations, the readers interested in more detailed data upon quantifying forces and stresses during organ formation in higher Vertebrates (mostly chicken embryos) are recommended to address the following references: Li et al., 2011; Filas et al., 2015.

Conclusions

We are well aware that the above described results obtained on amphibian embryos cannot guarantee that similar protocols can effectively work as applied to mammalian (human) embryonic tissues. By our knowledge, no such attempts has been as yet performed, although the mechanical backgrounds of organs formation in Amniota embryos are well substantiated [27]. In any case, a hard work by further elaboration and standartization of recommended procedures should be done. However these latter are in their essence so simple and the visible results so easily obtained that the cost of the required efforts seems to be not too high.

References

1. Beloussov LV, Dorfman JG, Cherdantzev VG. Mechanical stresses and morphological patterns in amphibian embryos. J Embryol Exp Morphol. 1975, 34(3): 559-574.

2. Kucera P, Monnet-Tschudi F. Early functional differentiation in the chick embryonic disc: interactions between mechanical activity and extracellular matrix. J Cell Sci Suppl. 1987, 8: 415-431.

3. Beloussov LV, Saveliev SV, Naumidi II, Novoselov VV. Mechanical stresses in embryonic tissues: patterns, morphogenetic role and involvement in regulatory feedback. Int Rev Cytol. 1994, 150: 1-34.

4. Ingber DE. Mechanical control of tissue morphogenesis during embryological development. Int J Devel Biol. (2006) , 50(2-3): 255-266.

5. Taber LA. Towards a unified theory for morphomechanics. Philos Trans A Math Phys Eng Sci. 2009, 367: 3555-3583.

6. Howard J1, Grill SW, Bois JS. Turing's next steps: the mechanochemical basis of morphogenesis. Nat Rev Mol Cell Biol. 2011, 12(6): 392-398.

7. Lev V Beloussov. Morphomechanics of Development (With a contribution by A Lipchinsky). Springer Cham Heidelberg NewYork Dordrecht London. 2015,195 P.

8. Farge E. Mechanical induction of Twist in the Drosophila foregut/stomodeal primordium. Curr Biol. 2003,13(16), 1365-1377.

9. McBeath R, Pirone DM, Nelson CM, Bhadriraju K, Chen CS. Cell shape, cytoskeletal tension and RhoA regulate stem cell lineage commitment. Dev Cell 2004, 6(4): 483-495.

10. Engler AJ, Sen S, Sweeney HL, Discher DE. Matrix elasticity directs stem cell lineage specification. Cell . 2006,126(4): 677-689.

11. Brunet T, Bouclet A, Ahmadi P, Mitrossilis D, Driquez B et al. Evolutionary conservation of early mesoderm specification by mechanotransduction in Bilateria. Nature Communications. 2013, 4:2821.

12. Beloussov LV, Luchinskaya NN, Ermakov AS, Glagoleva NS. Gastrulation in amphibian embryos, regarded as a succession of biomechanical feedback events. Int J Dev Biol V. 2006, 50(2-3): 113-122.

13. Beloussov LV, Lakirev AV, Naumidi II, Novoselov VV. Effects of relaxation of mechanical tensions upon the early morphogenesis of Xenopus laevis embryos. Int J Dev Biol. 1990, 34(4): 409-419.

14. ES Kornikova, EG Korvin-Pavlovskaya, LV Beloussov . Relocations of cell convergence sites and formation of pharyngula-like shapes in mechanically relaxed Xenopus embryos. Development, Genes and Evolution. 2009, 219(1): 1-10.

15. Chen CS, Mrksich M, Huang S, Whitesides GM, Ingber DE. Geometric control of cell life and death. Science.1997, 276: 1425-1428.

16. Evstifeeva, A Ju, Kremnev, SV, Beloussov LV. Changes of topology and geometry of Xenopus embryonic epithelia under relaxation of mechanical tensions. Ontogenez (Russ J Devel Biol). 2010, 41: 190-198.

17. Beloussov LV, Louchinskaia NN, Stein AA. Tension-dependent collective cell movements in the early gastrula ectoderm of Xenopus laevis embryos. Devel Genes and Evolution. (2000), 210(2): 92-104.

18. Troshina, TG, Glagoleva, NS, Beloussov LV. Statistical investigation of fast mechanodependent cell movements in deformed explants of Xenopus embryonic tissues. Ontogenez (Russ J Devel Biol). 2011, 42: 301-311.

19. Mansurov AN. Passive and active reactions of embryonic tissues of Xenopus embryos to the actions of external mechanical forces. Diss Cand Biol Sci. Moscow State University (in Russian). 2012.

20. Kremnyov SV, Troshina TG, Beloussov LV. Active reinforcement of externally imposed folding in amphibian embryonic tissues. Mechanisms of Development. 2012, 129(1-4): 51-60.

21. Kornikova ES, Troshina TG, Kremnyov SV, Beloussov LV. Neuro-mesodermal patterns in artificially deformed embryonic explants: a role for mechanogeometry in tissue differentiation. Devel Dynamics . 2010, 239(3): 885-896.

22. Von Dassow M, Davidson LA. Natural variation in embryo mechanics: gastrulation in Xenopus laevis is highly robust to variation in tissue stiffness. Dev Dyn. 2009, 238(1): 2-18.

23. Mansurov AN, Stein AA, Beloussov LV. A simple model for estimating the active reactions of embryonic tissues to a deforming mechanical force. Biomech. Modeling Mechanobiol. 2012, 11(8): 1123-1136.

24. Chien YH, Keller R, Kintner C, Shook DR. Mechanical strain determines the axis of planar polarity in ciliated epithelia. Curr.Biol. 2015, 25(21): 2774-2784.

25. Levental I, Georges PC, Janmey PA. Soft biological materials and their impact on cell function. Soft Matter. 2007, 3: 299-306.

26. Glagoleva NS, Beloussov LV, Stein AA, Luchinskaia NN. Quantitative study of regional and stage-specific reactions of Xenopus laevis embryonic tissues to mechanical load. Ontogenez (Russ. J. Devel Biol.). 2003, 34(4): 241-248

27. Taber L.A. Morphomechanics: transforming tubes into organs. Curr. Op. Gen. Devel. 2014, 27: 7-13.