Difference in the response to PIF/activin between animal caps excised from mid- or late blastula stages of *Xenopus laevis*

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Abstract. Animal caps from *Xenopus* embryos at Stage 7/8 cultured in salt solution, were capable of elongating, and formed various embryonic tissues including axial mesoderm, nervous tissues and pigmented retina. In contrast, animal caps from Stage 9 only developed into permanent blastulae. Following exposure to PIF/activin, however, such animal caps displayed morphogenetic movements. They formed tube-shaped embryoids that frequently had one or more tails but no head. We conclude that animal caps from Stage 9 produce more reliable results than those from Stage 7/8 because the latter are most likely contaminated with mesodermal cells.
Key words. Mesoderm induction; *Xenopus*; activin; axes formation.

Animal caps excised from of *Xenopus laevis* have been used to test mesoderm-inducing factors as well as to investigate embryonic axes formation (for a review see Melton[1]). Recently, a soluble peptide factor secreted by a mouse macrophage cell-line called PIF/activin[2] was found to induce embryoids with eyes and dorso-ventral axes[3] in animal caps excised from mid-blastula Stages 7/8[4].

We investigated whether PIF/activin could induce eyes in animal caps of the eyeless mutant Mexican salamander (*Ambystoma mexicanum*). For control purposes, we cultured animal caps of *Xenopus laevis* blastulae at Stage 7/8 in simple buffer solution or in such solution containing PIF/activin. We here report that animal caps of *Xenopus* excised from blastulae at Stage 7/8 were capable of embryonic tissue differentiation and of forming an antero-posterior axis. In contrast, animal caps of *Xenopus* blastulae prepared at Stage 9 and cultured in buffer solution formed blastulae only, but differentiated into tubular shaped embryoids only following exposure to PIF/activin.

Materials and methods

Xenopus eggs were obtained and artificially inseminated following standard procedures. The blastulae, either at Stage 7/8 or Stage 9, were dejellied using L-cystein. The animal caps from Stage 7/8 were of the size used by Sokol, Wong and Melton[3]. Animal caps from both stages were cultured in the way described by these authors, including the concentration of PIF/activin in the medium.

Photographs were taken roughly every 12 h and the circumference of the cultures measured using the software IMAGE (NIH) on a Macintosh IIci computer.

The statistical test Kruskal Wallis (SYSTAT) was used to determine the significance of the quantitative differences between the animal caps exposed to PIF/activin and the controls.

The embryoids were fixed and prepared for histology, or electron microscopy as reported earlier[5].

Results and discussion

Some animal caps from mid-blastula Stages 7/8 of *Xenopus laevis* cultured in simple buffer solutions were capable of differentiating into embryoids (fig. 1 A). They contained nervous tissues, pigmented retina, melanophores, mesenchyme as well as striated muscle and notochord (fig. 1 B). In animal caps also prepared from Stage 7/8 but treated with PIF/activin, these tissues again were present but eyes did not form (table, fig. 2).

Our results differ from the ones in which the effect of PIF on *Xenopus* blastulae at Stage 7/8 was first described[3]. These authors reported that untreated animal caps were only capable of forming ciliated blastulae. Following exposure to PIF/activin, however, such animal caps differentiated into various embryonic tissues including eyes.

We suspect that the discrepancy might be due to various amounts of contaminating mesodermal cells in animal caps from mid-blastula stages. The fate map of animal caps of mid-blastula stages indeed contains mesodermal cells[6]. This was demonstrated experimentally by Dale and Slack[7] by replacing animal caps of host blastulae with animal caps from fluorescently labeled donors (see their fig. 7).

Dale, Smith and Slack[6] cultured animal caps, for example from Stages 7.5 and 8 and found by using germ layer specific antibodies that only ectodermal components but not mesodermal structures formed. Judging from their figure 1 A, the sizes of the animal caps that they used, however, were roughly half the size from the ones used in this investigation.

Sudarwati and Nieuwkoop[8] reported that isolated animal caps from Stage 9 did not produce any mesoderm in vitro. Therefore, we prepared and cultured animal caps from Stage 9 in buffer solution and found that only ciliated blastulae formed. In contrast, such explants exposed to PIF/activin elongated and differentiated into embryoids (fig. 1 C, 1 E).

Compared to normal embryos, their anatomy was quite abnormal. For example, the notochord frequently alternated along the antero-posterior axis with musculature

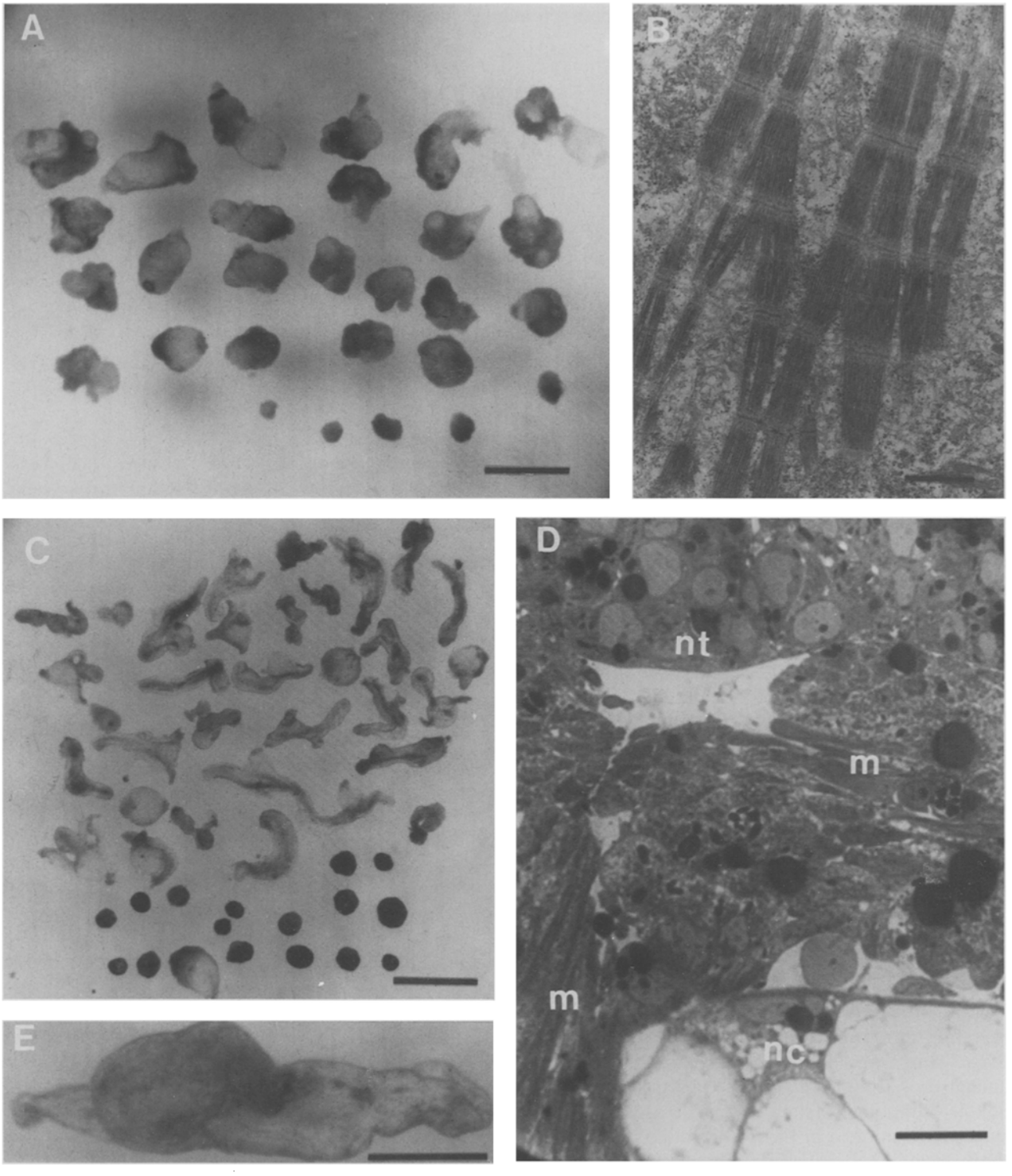

Figure 1. *A* Animal caps excised from blastulae between Stages 7/8 (4–5 h following artificial insemination), not exposed to PIF (controls) photographed after 72 h of development. Some of these embryoids showed spontaneous muscle contractions (twitching). Scale bar: 2 mm. *B* Fibers of striated musculature that developed in some of the control embryos illustrated in figure 1 A. Scale bar: 1 µm. *C* Animal caps excised from blastulae at Stages 9 (7–8 h following artificial insemination) and cultured for 66 h. The controls (the three rows on the bottom of fig. 1 C of the illustration) were cultured in buffered salt solution only. Scale bar: 2 mm. *D* Mid-sagittal section through an experimental embryoid fixed after 72 h of development. Striated musculature (m) is located between the notochord (nc) and the neural tube (nt). Scale bar: 50 µm. *E* Example of a typical 72-h-old embryoid that developed following exposure to PIF. Scale bar: 500 µm.

Comparison of elongation and differentiation capacities between animal caps excised from mid- or late blastula stages of *Xenopus laevis*

Exp.	Blastulae at Stage	No. of cultures		No. of elongating embryoids after 24 h		No. of antero-post. struct. after 72 h PIF			Cont.		
		PIF	Cont.	PIF (%)	Cont. (%)	p. ret.	c.g.	tails	p. ret.	c.g.	tails
1	7/8	38	28	20 (57)	11 (39)	0	10	13	1	7	6
2	7/8	41	45	26 (63)	15 (33)	2	19	18	2	10	14
3	9	39	27	31 (79)	0 (0)	0	28	25	0	0	0
4	9	65	42	61 (94)	0 (0)	0	45	42	0	0	0

p.ret.: pigmented retina only (not complete eyes). c.g.: cement glands.

and mesenchyme. Occasionally, two or more notochords were present in the same cross section. We confirm that striated musculature often formed a cylinder around such notochord fragments [3]. As a consequence, striated musculature frequently separated the neural tube from the notochord (fig. 1 D). The neural tissues also formed tubular fragments, isolated from one another by mesenchyme and striated musculature. This situation suggests to us that various mesodermal tissue components might be stimulated to differentiate along different pathways due to local variations in the concentration of one and the same factor(s) [9]. Histological analysis further revealed the presence of embryonic mesenchyme, melanophores, and blood islands. Along the antero-posterior axis, neural tissues occasionally differentiated medially, on opposite sides of the cylindrical musculature that surrounded the notochord. In our opinion, this indicates a lack of dorso-ventral information in the embryoids. These findings counterbalance results suggesting that the dorsal and ventral halves of animal caps from blastulae at Stage 8/8.5 already have an established dorso-ventral axial system [10].

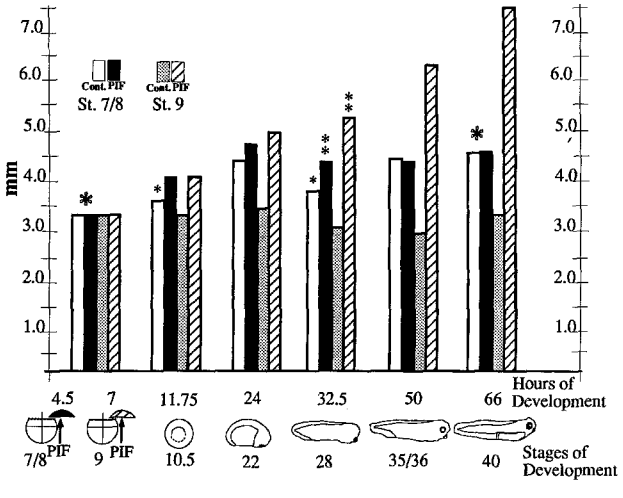

Figure 2. Lengthening of animal caps (excised from mid-blastula or late blastula stages) following exposure to activin/PIF. Photographs of controls and the experimental group were taken approximately every 12 h. The circumference of the animal cap cultures were measured with the help of the software IMAGE (NIH) on a Macintosh IIci computer. The mean diameter of the animal caps was then used to find the points of intersection with the circle representing the mean diameter of the complete blastulae (see the illustration of the animal caps). The Kruskal Wallis statistical test (SYSTAT) was used to determine the significance of the quantitative differences between the animal caps exposed to PIF and the controls. * Significant difference between the circumference of the cultures (mean) at the beginning and after 66 h of culturing ($p < 0.05$). * Significant difference between controls and animal caps exposed to PIF ($p < 0.05$). ** Significant difference between the circumference of animal caps from Stage 7/8 and Stage 9. n = 38

In a quantitative evaluation we found that animal caps from Stages 7/8 exposed to PIF/activin elongated faster than controls (fig. 2). This could be detected and measured as soon as 4 h after exposure (fig. 2: 11.75 h). However, the animal pole explants form Stage 9 similarly treated, elongated much faster than the ones from Stage 7/8 (fig. 2: 32.5 h).

We confirm that the first detectable reaction of the animal cap tissues to PIF/activin was morphogenetic movement [10]. The explants first healed into spheres and then started to elongate as soon as 4 h after exposure to PIF/activin without ever forming a blastopore (fig. 2: 11.75 h). Interestingly, this elongation was synchronous with the gastrulation movements in normal control embryos; i.e., there was no detectable delay.

In our view, PIF/activin is not primarily a mesoderm inducer but evokes morphogenetic movements in the blastocoel roof necessary for gastrulation and neurulation (for a discussion of results obtained with the chick embryo see Mitrani et al. [11]). This interpretation is supported by the observation that activin B is first expressed at the late blastula stage [2], a time when mesoderm has already been induced [12] (for a review see Smith [9]).

In isolated animal caps, PIF/activin might turn on a primary regulatory gene(s) which controls morphogenetic movements and only secondarily the production of factors necessary for the formation of the basic embryonic tissues. From this perspective, tail formation in these embryoids, for example, would be the result of a compensatory, genetic cascade of sequential gene activation.

In conclusion, it might be advantageous for various bioassays to use animal caps from Stage 9 *Xenopus laevis* embryos instead of blastulae at Stage 7/8. They are less likely to be contaminated with mesodermal cells.

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Bdelloid rotifers in Dominican amber: Evidence for parthenogenetic continuity

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Abstract. Recently discovered representatives of the Class Bdelloidea from Tertiary amber from the Dominican Republic represent the oldest known fossils of the Phylum Rotifera. Assuming that the fossil bdelloids had a similar mode of reproduction as present day members of the Class (apomictic thelytoky), then contrary to current thought, some lines of parthenogenetic organisms are not doomed to an early extinction and have evolved built-in mechanisms for genetic diversity.
Key words. Fossil rotifers; Bdelloidea; Tertiary amber; Dominican Republic.

Representatives of the Phylum Rotifera are poorly represented in the fossil record, being known only from the Holocene (8000 years and younger)[1]. A piece of amber from the Dominican Republic was found to contain a population of 22 rotifers as well as another organism that could be a rotifer but cannot be assigned with certainty to this or any other Phylum. At least 18 of the 22 rotifers could be assigned to the Order Bdelloida. All of these animals were associated with the cap of the fossil gilled mushroom *Coprinites dominicana* Poinar and Singer[2]. The total fossil assemblage represents a palaeobiocoenosis or 'fossil community'.

The amber containing the fossils originated from the La Toca mine, located between Santiago and Puerto Plata in the Cordillera Septentrional of the Dominican Republic. This mine is in the Altimira facies of the El Mamey Formation (upper Eocene), which is shale-sandstone interspersed with a conglomerate of well-rounded pebbles[3]. Differences in the magnitudes of absorption peaks in nuclear magnetic resonance spectra of the exomethylene group of amber[4] from different mines in the Dominican Republic were used to calibrate the ages of the various mines, with the age (20 million to 23 million years); (based on foraminifera counts) of the Palo Alto mine used as a standard[5]. The ages of various pieces of Dominican amber ranged from 15 million to 40 million years with that from the La Toca mine being the oldest, some 35 million to 40 million years old (lower Oligocene to upper Eocene). This age is within the independent dating reported by Cepek[6] who gave a range of 30 to 45 million years for "La Toca" mine.

The piece of amber containing the fossils (AF-9-11) had all the visual characteristics of natural Dominican amber. A series of chemical and physical tests[7] performed on a small portion of the amber piece verified that it was authentic. The piece of yellow transparent amber containing the rotifers weighed 0.5 g and was elliptical in shape, 9 mm long and 6 mm wide. It is deposited in the Poinar collection of Dominican amber maintained at the University of California, Berkeley.

All of the bdelloid rotifers were contracted so that the corona and foot were unrecognizable. Because it was impossible to observe the number of toes, the stomach lumen and the shape of the trochi and the mastax, it was not possible to place them in any existing family with certainty. The size of the bdelloid rotifers ranged from 126 to 504 μm in length (av = 266 μm) and from 76 to 200 μm in width (av = 149 μm) (N = 18). The specimen shown in figure 1 (L = 360 μm; W = 189 μm) shows the tail incompletely extended and covered with small spines arranged in parallel files (fig. 2). As can be seen from a portion of the trunk surface that is free from detritus, the trunk was also covered with transverse rows of posteriorly directed small spines (fig. 3). Similar patterns of cuticular ornamentation occur in representatives of the genera *Habrotrocha* and *Scepanotrocha* of the family Habrotrochidae and *Macrotrachela* and *Rotaria* of the family Philodinidae.

Figure 4 shows the specimen that could also be a rotifer but does not resemble any extant forms. It is unique in the foot and trunk possessing large spines hinged at their bases. Although a few bdelloids have trunk spines, none