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Short Contribution

Amphibian embryos as a model system for organ engineering: *in vitro* induction and rescue of the heart anlage V

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ABSTRACT Beating hearts can be induced under *in vitro* conditions when the dorsal blastopore lip (including the zone of Spemann organizer) is treated with Suramin. In contrast, untreated organizer forms dorsal mesodermal derivatives as notochord and somites. When those *in vitro* produced heart precursor tissues are transplanted ectopically in the posterior trunk area of early larvae, secondary beating heart structures will be formed. Furthermore, the replacement of the heart primordium of the host embryo by heart tissue induced under *in vitro* conditions will result in the rescue of the heart anlage. This model could be a valuable tool for the study of the multi-step molecular mechanisms of heart structure induction under *in vitro* conditions and vasculogenesis after transplantation into the host embryo.

KEY WORDS: *in vitro heart induction, heart anlage rescue, ectopic heart, transplantation, organ engineering, Suramin*

Rhythmic beating heart structures can be received under *in vitro* conditions (Figs. 1,2) when isolated dorsal blastopore lip is treated with Suramin (Grunz, 1992). In contrast, untreated isolated organizer will form dorsal mesodermal structures like notochord and somites (Spemann and Mangold, 1924). It could be shown, by heart specific genes *(troponin or XNKx2-5)* as molecular markers (Drysdale *et al.*, 1994; Tonissen *et al.*, 1994; Patterson *et al.*, 1998), that the Spemann organizer forms heart structures in 100% of the cases after Suramin treatment (Fig. 2).

In an attempt to show that such heart tissue could function normally *in vivo*, in a first trial we transplanted Suramin-treated dorsal blastopore lip into the posterior trunk area of stage 25 larvae (early tailbud stage) (see Experimental Procedures, Fig. 1). The implanted tissue is integrated easily into the surrounding tissue of the host embryo. All explants (20 cases) formed heart-like structures after several days' culture (Fig. 3E,F). In four larvae the additional ectopic heart structures showed strong rhythmic contractions, which were also documented by videotape monitoring. Histological sections could show that the ectopic structures in all cases indeed consisted of heart differentiations (Fig. 4). Transplanted untreated dorsal blastopore lip differentiated into notochord and somites (not shown).

In a further series we replaced the original heart primordia of host embryos (stage 20, compare with Fig. 2A) by Suramin treated dorsal blastopore lip (Fig. 1). In 5 of 10 cases the transplantation resulted in beating hearts and nearly normal larvae (Fig. 3A,C). In contrast, all larvae (15 of 15) of the control series (heart anlage removed) developed into hypertrophic larvae without heart structures (Fig. 3B,D). The hypertrophy can be explained by the fact that the pronephric system correlated with the heart function does not work properly. Similar effects were observed in a kidney organ engineering system after bilateral pronephrectomy (Chan *et al.*, 1999).

Heart structures, even the ectopic ones, are very similar to the normal heart of this developmental stage (Fig. 4). The heart shows rhythmic contractions. Both larvae with ectopic heart structures and rescued heart primordium are swimming around in the culture vessel like normal larvae. A further indication for the successful transplantation of the *in vitro* cultured heart tissue and rescue of the heart anlage could be monitored by the presence of an additional cement gland (Figs. 3A,C,F and 4E,F). In an earlier paper, we could show that dorsal blastopore lip treated with Suramin contains cement gland in 100% of the cases in addition to the heart structures (Grunz, 1992). This is of certain interest since all neural structures are suppressed, in Suramin-treated Spemann organizer (Fig. 2). Apparently, the cement gland gene expression, considered as a week neural induction, is not affected by the Suramin-treatment (Sive and Bradley, 1996;

Abbreviations used in this paper: cg, cement gland; b, brain; no, notochord; nh, normal heart; eh, ectopic heart; ot, otic vesicles; gi, gill area; f, fin; h, heart; neu, neural tube; so, somites.

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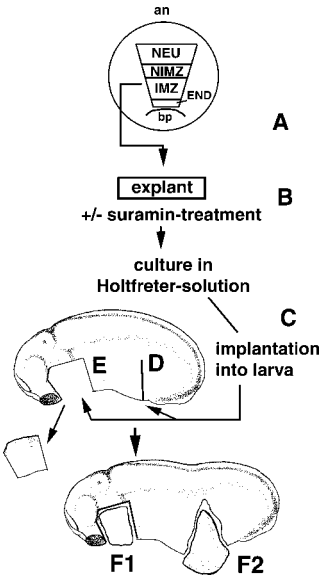

Fig. 1. *In vitro* induction of heart structures and transplantation into larvae. (A) *Isolation of dorsal blastopore lip. The area includes neuroectoderm (NEU), non involuting mesoderm (NIMZ), involuting mesoderm (IMZ) and headendoderm (END) and underlying mesoderm.* **(B)** *The explant was treated with or without Suramin (150 μM, 4 h) and cultured in Holtfreter-solution until siblings reached stage 20 (rescue experiments, see Fig. 2A) or tailbud stage (st. 25, Nieuwkoop and Faber, 1956).* **(C)** *The slit in the posterior trunk area of the larva (D) or the extirpation of the heart anlage (E) were performed with fine Spemann glass needles, followed by insertion of the explant into the anterior (F1, rescue experiment) or posterior (F2, ectopic transplantation) trunk area.*

Gammill and Sive, 1997; Aberger *et al.*, 1998). The shift of the pattern of the Spemann organizer by Suramin from dorsal to ventral structures can be explained by activation of BMP-4 expression (Fainsod *et al.*, 1994). The dorsal blastopore area in normogenesis is free of BMP-4 (De Robertis *et al.*, 1997). In contrast to the ventral side, dorsalizing and neuralizing genes are expressed within the zone of the Spemann organizer (Grunz, 1997). Such genes encode either transcription factors or secreted proteins. They include the homeobox gene *goosecoid (gsc)* (Cho *et al.* 1991), the T-box genes *brachyury* (Smith *et al.*, 1991) and *eomesodermin* (Ryan *et al.*, 1996) as well as the zinc finger gene *Xegr-1* (Panitz *et al.*, 1998). Furthermore, the secreted factors noggin (Smith and Harland, 1992), Xnr3 (Smith *et al.,* 1995) and Chordin (Sasai *et al.*, 1994) are exclusively expressed in this area.

The Suramin-treatment of isolated dorsal blastopore lip will result in the expression of BMP-4, which is an antagonist of several organizer-specific genes (Fainsod *et al.*, 1994). However, BMP-4 or

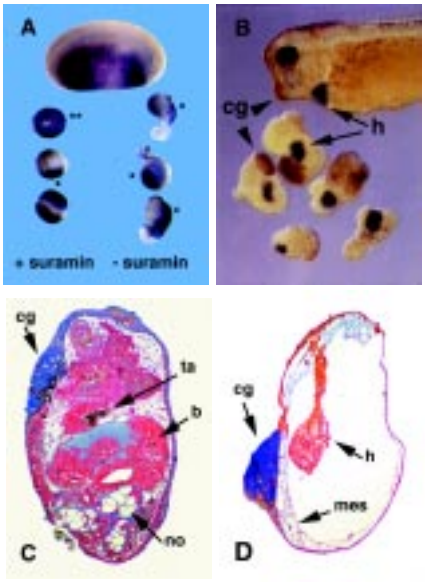

Fig. 2. Isolated dorsal blastopore lip treated with or without Suramin. **(A)** *Dorsal blastopore lip treated with or without Suramin was fixed in HEMFA when sibling larvae had reached stage 20 (top of the panel). The pan-endodermal gene marker endodermin can be detected in both series (one asterisk= view from the side; two asterisks= view from the top.* **(B)** *Whole-mount in situ hybridization of Suramin treated Spemann organizer (courtesy, Tom Drysdale and Paul Krieg). The heart specific marker can be identified in all cases. Note that also simultaneously cement gland can be observed (compare with histology in D).* **(C)** *Untreated dorsal blastopore lip including ectoderm (Spemann organizer) differentiates into notochord (no), brain structures (b) with a rudimentary eye (ta= tapetum) and cement gland (cg).* **(D)** *Dorsal blastopore lip treated with Suramin (150 μM, 4 h) has differentiated into a heart tube and mesenthelium.*

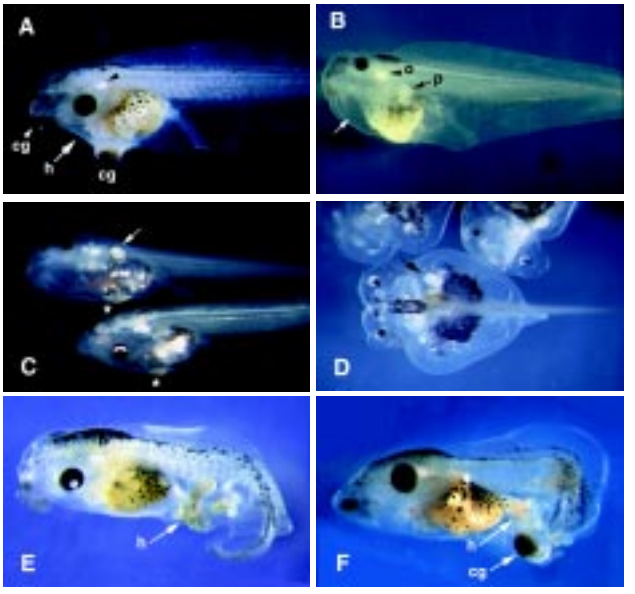

Fig. 3. Larvae after rescue of the heart anlage or larvae with additional ectopic hearts. (A) *The heart primordium of this larva was removed at stage 20 and was replaced by Suramin-treated blastopore lip (rescue experiment). The larva shows a rhythmic beating heart (h) and an additional cement gland (cg).* **(B)** *Control larva. The heart anlage of this larva was removed at stage 20. No regeneration of the heart (zone at the white arrow) took place in this hypertrophic larva with enlarged pronephric area (p).* **(C)** *Same series as in (A), rescue experiment. The 12 day old larvae have a rhythmic beating heart and are comparable to normal larvae.* **(D)** *Same series as in (B). The 12 day old larvae without heart show severe hypertrophy with enlarged pronephros (arrow). The heart anlage of these larvae was removed at stage 20. No beating heart (heart- free zone, see asterisk) could be identified.* **(E)** *Suramin-treated dorsal blastopore lip was implanted in the posterior trunk area. The larvae (stage 47) showed rhythmic contractions of the ectopic heart (h).* **(F)** *In another larva, also clearly rhythmic beating heart structures could be observed in addition to the beating normal heart. A second cement gland has differentiated together with the heart structure. o= otolith.*

BMP-2 alone cannot support ectopic cardiac differentiation (Dale *et al.*, 1992; Clement *et al.*, 1995; Patterson *et al.*, 1998). Important for heart development is that some head endomesoderm is included in the isolated dorsal blastopore lip. It is known that Cerberus mRNA expressed in this area will induce secondary head structures including the heart, when injected into the ventral side of early cleavage stages (Bouwmeester *et al.*, 1996). Heart structures will not form when animal caps are isolated from Cerberus injected embryos. Cerberus appears to be capable of regulating *NKx2-5* both in the larvae and in

animal caps. However, cardiac differentiation markers are not observed in animal caps (Bouwmeester *et al.*, 1996). This holds true also for animal caps treated with Cerberus protein (Piccolo *et al.*, 1999). This indicates that several additional factors are responsible for heart induction (Okada, 1954; Sasai *et al.*, 1996), which are present in the normal larvae only. The importance of endoderm for heart development and the mutual interaction of the heart primordium with the Spemann organizer could be shown by Nascone and Mercola (1995). The molecular mechanisms leading to the complex 3-dimensional heart or liver organs are poorly understood, because they consist of a multi-step process. Most of these steps include cell interactions and induction. So, experiments with the inducer Activin, a growth factor of TGFβ-protein-superfamily, turned out to be a valuable tool to study heart development under *in vitro* conditions. Depending on the concentration and incubation time, Activin induces ventral and/or dorsal mesoderm. At higher concentrations Activin also induces endoderm. When at a certain concentration both mesodermal and endodermal predetermination takes place, heart structures will be formed (Grunz, 1983). In a different approach we could induce liver tissue under *in vitro* conditions (Minuth and Grunz, 1980). Asashima and collaborators (Ariizumi *et al.*, 1996) received beating hearts when they treated urodelian animal caps with relative high Activin concentrations (100 ng/ml). Under these experimental conditions both mesoderm and endoderm structures will be induced. Experiments in our lab are in preparation to show that heart structures induced under *in vitro* conditions by Activin will also develop into beating hearts after implantation into normal larvae. Interestingly, animal caps treated simultaneously with Activin and Suramin will not form heart structures, but blood cells and mesenthelium only, the very ventral mesodermal structures (Oschwald *et al.*, 1993). These results support the view that the combined presence of both endoderm and organizer is necessary to induce heart in ventral mesoderm (Nascone and Mercola, 1995). Using the pan-endoderm gene marker, *endodermin* (Sasai *et al.*, 1996), it could be shown that in both untreated and Suramin-treated dorsal blastopore lip endodermal tissue is still present (Fig. 2A). This means that Suramin prevents the formation of dorsal mesodermal structures (notochord and somites) and neural structures, but does not inhibit the expression of the endoderm marker.

The results presented above show that heart structures induced by *in vitro* culture of Spemann organizer in Suramin will form ectopic beating hearts after transplantation into normal larvae and are able to rescue the original heart primordium. This amphibian model system could be important to study the integration of heart structures induced under *in vitro* condition and the vascular development. Despite intensive research in many laboratories, the molecular mechanisms responsible for the multi-step process leading to the 3-dimensional heart organ, are still unknown (reviewed by Neff *et al.*, 1996). Further studies with *in vitro* induced heart and liver by Activin will be useful to establish organ engineering models to analyze factors (genes and their products) responsible for the development of the cardiac and hepatic system.

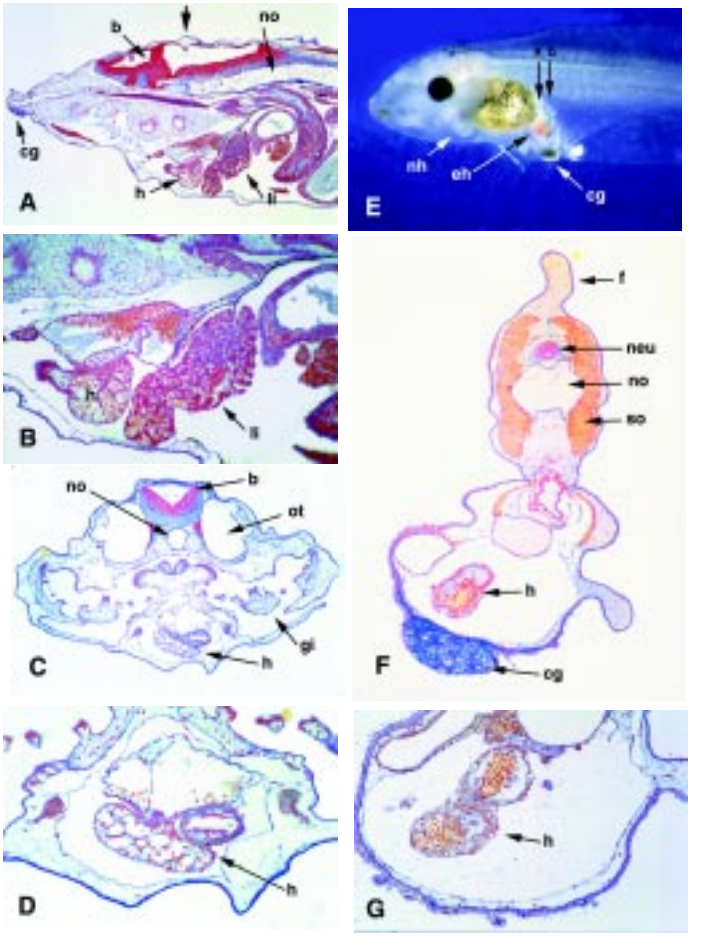

Fig. 4. Comparison of heart structures of the normal larva (stage 47) with ectopic heart structures of the transplanted *in vitro* induced heart structures. (A-D) *Normal heart.* **(E-G)** *Ectopic heart.* **(A)** *Sagittal section of a normal larva (stage 47).* **(B)** *Higher magnification of the section shown in (A).* **(C)** *Transversal section of a stage 47 in the heart region (see arrow head in (A).* **(D)** *Higher magnification of (C).* **(E)** *Larva (stage 47) with ectopic heart.* **(F)** *Transversal section of larva shown in (E) in the heart region (arrow b).* **(G)** *Higher magnification of a similar section, shown in (F), but more anterior (see arrow a) cg, cement gland; b, brain; no, notochord; nh, normal heart; eh, ectopic heart; ot, otic vesicles; gi, gill area; f, fin; h, heart; neu, neural tube; so, somites; li, liver.*

Experimental Procedures

Xenopus embryos

Xenopus laevis eggs were obtained by injecting female frogs with 1000 IU human chorion gonadotropin (Schering AG, Berlin, Germany) prior to *in vitro* fertilization. The embryos were raised in Steinberg solution (58.18 mM NaCl, 0.67 mM $MnCl_2$, 0.34 mM Ca $(NO_3)_2$, 0.8 mM $MgSO_4$; pH 7.4) up till stage 10 according to Nieuwkoop and Faber (1956). The jelly coat was removed by treatment with 3.5% cysteiniumchloride (pH 7.4) for 5 to 7 min depending on the temperature of the solution. The embryos were rinsed several times in Holtfreter solution to which penicillin/streptomycin had been added. The vitelline membrane was removed mechanically with fine watchmakers' forceps.

Suramin treatment

Heart structures were induced as described elsewhere (Grunz, 1992). In short, dorsal blastopore lips (Spemann organizer) from early gastrulae (stage 10-10.5, Nieuwkoop and Faber, 1956) were incubated with or without 150 mM Suramin (Naganol®, Bayer AG) for 4 h. The dorsal blastopore lip contained parts of ectoderm and the presumptive head mesendoderm (Fig. 1). After transfer of treated dorsal blastopore lip into normal Holtfreter solution, the tissue was cultured until normal larvae had reached early tailbud-stage (stage 25) for ectopic hearts transplantation or stage 20 for the rescue experiments.

Implantation of presumptive heart into the host embryos

Untreated and Suramin treated blastopore lips were transplanted into the posterior trunk area of host larvae (stage 25, Fig. 1). In a second series (rescue experiments), the heart anlage of earlier embryos (stage 20, compare with Fig. 2A) was extirpated and replaced by *in vitro* induced heart primordium. The grafts were quickly integrated and the larvae were raised until stage 47. The rhythmic contractions of several larvae were very intensive and were documented on videotape. Macroscopic and microscopic documentation was done with a Zeiss Axioplan-microscope, Zeiss-Stereomicroscope Stemi 2000-CS, a Leitz Orthomat, Video Camera (Sony DXC-9100P) and S-VHS-Video Recorder (Sony SVT-S3050P). Histology and whole-mount *in situ* hybridization were performed as described elsewhere (Grunz, 1983; Oschwald *et al*., 1991; Chen *et al*., 1999). The color slides were scanned with a Nikon-Scanner LS-1000. Documentation was performed with Adobe Photoshop and a Kodak 8650 PS printer.

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References

ABERGER, F., WEIDINGER, G., GRUNZ, H. and RICHTER, K. (1998). Anterior specification of embryonic ectoderm: the role of the *Xenopus* cement gland-specific gene *XAG-2. Mech. Dev. 72:* 115-130.

ARIIZUMI, T., KOMAZAKI, S., ASASHIMA, M. and MALACINSKI, G.M. (1996). Activin treated urodele ectoderm: a model experimental system for cardiogenesis. *Int. J. Dev. Biol. 40:* 715-718.

BOUWMEESTER, T., KIM, S.H., SASAI, Y., LU, B. and DE ROBERTIS, E.M. (1996). Cerberus is a head-inducing secreted factor expressed in the anterior endoderm of Spemann's organizer. *Nature 382:* 595-601.

CLEMENT, J.H., FETTES, P., KNÖCHEL, S., LEF, J. and KNÖCHEL, W. (1995). Bone morphogenetic protein 2 (BMP-2) in the early development of *Xenopus laevis*. *Mech. Dev. 52:* 357-370.

CHAN, T., ARIIZUMI,T. and ASASHIMA, M. (1999). A model system for organ engineering: transplantation of *in vitro* induced embryonic kidney. *Naturwissenschaften 86:* 224-227.

CHEN, Y.L., HOLLEMANN, T., GRUNZ, H. and PIELER, T. (1999). Characterization of the Ets-type protein ER81 in *Xenopus* embryos. *Mech. Dev. 80:* 67-76.

CHO, K.W.Y., BLUMBERG, B., STEINBEISSER, H. and DE ROBERTIS, E.M. (1991). Molecular Nature of Spemanns Organizer - The Role of the *Xenopus* Homeobox Gene *goosecoid. Cell 67:* 1111-1120.

DALE, L., HOWES, G., PRICE, B.M.J. and SMITH, J.C. (1992). Bone Morphogenetic Protein-4 - A Ventralizing Factor in Early *Xenopus* Development. *Development 115:* 573-585.

DE ROBERTIS, E.M., KIM, S., LEYNS, L., PICCOLO, S., BACHILLER, D., AGIUS, E., BELO, J.A., YAMAMOTO, A., HAINSKIBROUSSEAU, A., BRIZUELA, B., WESSELY, O., LU, B. and BOUWMEESTER, T. (1997). Patterning by genes expressed in Spemann's organizer. *Cold Spring Harbor Symp. Quant. Biol. 62:* 169-175.

DRYSDALE, T.H., TONISSEN, K., PATTERSON, K., CRAWFORD, M. and KRIEG, P.A. (1994). Cardiac Troponin I Ia a Heart-Specific Marker in the *Xenopus* Embryo: Expression during Abnormal Heart Morphogenesis. *Dev. Biol. 165:* 432-441.

FAINSOD, A., STEINBEISSER, H. and DE ROBERTIS, E.M. (1994). On the function of BMP-4 in patterning the marginal zone of the *Xenopus* embryo. *EMBO J. 13:* 5015-5025.

GAMMILL, L.S. and SIVE, H. (1997). Identification of otx2 target genes and restrictions in ectodermal competence during *Xenopus* cement gland formation. *Development 124:* 471-481.

GRUNZ, H. (1983). Change in the differentiation pattern of *Xenopus laevis* ectoderm by variation of the incubation time and concentration of vegetalizing factor. *Roux's Arch. Dev. Biol. 192:* 130-137.

GRUNZ, H. (1992). Suramin Changes the Fate of Spemann's Organizer and Prevents Neural Induction in *Xenopus-Laevis. Mech. Dev. 38:* 133-142.

GRUNZ, H. (1997). Neural induction in amphibians. In *Current Topics in Developmental Biology,* (eds. R.A. Pedersen and G.P. Schatten), Academic Press, San Diego, pp 191-228.

MINUTH, M. and GRUNZ, H. (1980). The formation of mesodermal derivates after induction with vegetalizing factor depends on secondary cell interactions. *Cell Differ. 3:* 229-238.

NASCONE, N. and MERCOLA, M. (1995). An inductive role for the endoderm in *Xenopus* cardiogenesis. *Development 121:* 515-523.

NEFF, A.W., DENT, A.E. and ARMSTRONG, J.B. (1996). Heart development and regeneration in urodeles. *Int. J. Dev. Biol. 40:* 719-725.

NIEUWKOOP, P.D. and FABER, J. (1956). *Normal table of Xenopus laevis* (Daudin). North Holland, Amsterdam.

OKADA, T.S. (1954). Experimental studies on the differentiation of endodermal organs in amphibians. II.Differentiation potencies of the presumptive endodermin the presence of the mesodermal tissue. *Mem. Coll. Sci. Univ. Kyoto (ser.B.) 21:* 7-14.

OSCHWALD, R., CLEMENT, J.H., KNÖCHEL, W. and GRUNZ, H. (1993). Suramin Prevents Transcription of Dorsal Marker Genes in *Xenopus-laevis* Embryos, Isolated Dorsal Blastopore Lips and Activin-A Induced Animal Caps. *Mech. Dev. 43:* 121-133.

OSCHWALD, R., RICHTER, K. and GRUNZ, H. (1991) Localization of nervous system specific class II (β-tubulin gene in *Xenopus laevis* by whole mount *in situ* hybridization. *Int. J. Dev. Biol. 35:* 399-405.

PANITZ, F., KRAIN, B., HOLLEMANN, T., NORDHEIM, A. and PIELER, T. (1998). The Spemann organizer-expressed zinc finger gene Xegr-1 responds to the MAP kinase/Ets-SRF signal transduction pathway. *EMBO J. 17:* 4414-4425.

PATTERSON, K.D., CLEAVER, O., GERBER, W.V., GROW, M.W., NEWMAN, C.S. and KRIEG, P.A. (1998). Homeobox genes in cardiovascular development. *Curr. Top. Dev. Biol. 40:* 1-44.

PICCOLO, S., AGIUS, E., BHATTACHARYYA, S., GRUNZ, H., BOUWMEESTER, T. and DE ROBERTIS, E.M. (1999). The head-inducer Cerberus is a multifunctional antagonist of Nodal, BMP and Wnt signals. *Nature 397:* 707-710.

RYAN, K., GARRETT, N., MITCHELL, A. and GURDON, J.B. (1996). *Eomesodermin*, a key early gene in *Xenopus* mesoderm differentiation. *Cell 87:* 989-1000.

SASAI, Y., LU, B., PICCOLO, S. and DE ROBERTIS, E.M. (1996). Endoderm induction by the organizer-secreted factors chordin and noggin in *Xenopus* animal caps. *EMBO J. 15:* 4547-4555.

SASAI, Y., LU, B., STEINBEISSER, H., GEISSERT, D., GONT, L.K. and DE ROBERTIS, E.M. (1994). *Xenopus* chordin: A novel dorsalizing factor activated by organizer-specific homeobox genes. *Cell 79:* 779-790.

SIVE, H. and BRADLEY, L. (1996). A sticky problem: The *Xenopus* cement gland as a paradigm for anteroposterior patterning. *Dev. Dynamics 205:* 265-280.

SMITH, J.C., PRICE, B.M.J., GREEN, J.B.A., WEIGEL, D. and HERRMANN, B.G. (1991). Expression of a *Xenopus* Homolog of *Brachyury (T)* Is an Immediate-Early Response to Mesoderm Induction. *Cell 67:* 79-87.

SMITH, W.C. and HARLAND, R.M. (1992). Expression Cloning of noggin, a New Dorsalizing Factor Localized to the Spemann Organizer in *Xenopus* Embryos. *Cell 70:* 829-840.

SMITH, W.C., MCKENDRY, R., RIBISI, S. and HARLAND, R.M. (1995). A *nodal*-related gene defines a physical and functional domain within the Spemann organizer. *Cell 82:* 37-46.

SPEMANN, H. and MANGOLD, H. (1924). Über Induktion von Embryonalanlagen durch Implantation artfremder Organisatoren. *Roux's Arch. Dev. Biol.. 100:* 599-638.

TONISSEN, K.F., DRYSDALE, T.A., LINTS, T.J., HARVEY, R.P. and KRIEG, P.A. (1994). Xnkx-2.5, a *Xenopus* Gene Related to *Nkx-2.5* and *Tinman* - Evidence for a Conserved Role in Cardiac Development. *Dev. Biol. 162:* 325-328.

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