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Evolutionary analysis of genes involved in cement gland development in anurans

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Abstract

The cement gland (CG) is a transient organ, found in most anuran embryos and early larvae and located normally on the front of the head. Its sticky secretion allows newly hatched larvae to attach to the egg jelly or to another support later and remain hidden and stationary until feeding starts. Our ultrastructural studies showed that prominence structure of the CG in some species exists, but is lacking in some others. Previous work has shown that a large number of genes have a role in CG development in *Xenopus laevis.* The aim of the present study is to find out whether the loss of cement gland formation for those species studied here occurs because of missing genes or for other reasons.

In order to test whether some of these genes are present in other anuran species, especially in those where the CG does not form, genomic DNAs were examined for sequence similarity by low stringency hybridization. Sequences from three different genes with a role in controlling CG development in *Xenopus* (*otx-2, xcg-1* and *xag-1*) were individually hybridised with genomic DNA of four species of anurans (*X. laevies, Leptodactylus fuscus, Phyllomedusa trinitatis* and *Physalaemus pustulosus*) and one species of rodent (*Muss musculus domesticus* strain C57Bl/ 6). The results showed that *Xenopus* probes can detect the presence of potential homologues of all three genes in the different species. For the two genes most specifically involved in CG development, *xcg-1* and *xag-1*, both are clearly present, even in the two species which lack CG development, though in one of these, *P. trinitatis*, *xag-1* shows considerable difference from the other species. At this stage, we can conclude that the missing cement gland for those species studied here is not due to a lack of the genes responsible for the gland development.

In order to carry this work further, in situ hybridisation should be used to determine the actual expression patterns of these genes.

Keywords: Cement gland; gene controlling; evolution, amphibian; transient organ; morphogenesis

1. Introduction

Amongst the Chordata, sticky organs with similar position and probably a similar function to the cement gland of anurans are seen in some protochordates and in the embryos of some teleost fish [1, 2]. Pennati et al. [3] found that PhAG from the ascidian adhesive papillae has a considerable similarity to XAG, which is expressed in the cement and hatching glands of *Xenopus laevis*. In amniotes the cement gland is not present; however, the homeodomain gene *Xotx2* that is expressed in the *Xenopus* cement gland is also expressed in the buccopharyngeal region of mice [4].

Sive and Bradley [5] proposed the cement gland as a developmental paradigm in amphibians since it delineates the anterior end of the embryo and serves as a highly visible indicator of anterior induction. Many of the signals involved in neural induction also seem to be involved in cement gland development. Since cement gland differentiation occurs many hours before neurogenesis and involves fewer steps, analysis may be simpler.

Nokhbatolfoghahai and Downie [6] identified five different general morphologies of CG among 20 anuran species investigated, which differ essentially on whether or not the gland separates into two parts, the stage at which this happens, and the shapes of the complete and separated structures.

Some frogs that have embryos with a long intracapsular developmental period and that hatch at later stages may have very transient adhesive organs. Frog embryos that do not feed at the larval stage (or have direct development), and those species with embryos that are not aquatic, do not form a cement gland [7]. It is a matter of

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evolutionary and developmental interest to investigate how CG development has been lost in some anurans [8]. The lack of CG in the terrestrial foam nesting *Leptodactylus fuscus* compared to other leptodactylids foam nesting with CG lead the larvae to remain in the nest after hatching and until their tails were well grown. The post-hatching period in the nest is a normal part of development, suggesting an evolutionary reproductive transition to the terrestrial development within the leptodactylids [9].

Sive et al. [10] suggested that the CG developed where low levels of inducers were present. Drysdale and Elinson [11] showed several tissues such as yolky endoderm, chordal mesoderm and prechordal mesoderm by inhibitory or stimulatory signals are able to suppress or induce cement gland formation. Sive and Bradley [5] also showed that both stimulatory and inhibitory signals could determine cement gland formation at the appropriate site.

Jamrich and Sato [12] and also Sive et al. [10] isolated several different cDNA clones from genes that are early markers of CG development in *X. laevis*. The clones fall into three sets, according to spatial localization. The first set with three members (XCG-1, -2, and -3, for *Xenopus* Cement Gland) are expressed exclusively in the developing and mature cement gland. As XCG-1 is 10-fold more abundant than XCG-2 or XCG-3, this RNA was used as a cement gland specific marker. *xcg-1*: encodes a mucin- like protein [5] expressed before the CG is morphologically evident, but later the expression pattern is coincident with CG.

The second RNA class (XA-1, for *Xenopus* Anterior) was expressed uniformly in the CG primordium and anterior neural plate of late gastrula and early neurula. The third set of transcripts (XAG-1, XAG-2, and XAG-3, for *Xenopus* Anterior Gradient) is first detectable in the early gastrula stage 10/11 [13] and persists after the cement gland has degenerated. *xag-1*: encodes a transcription factor. Expression pattern is very similar to *xcg-1*. Thompson and Weigel [14] found that *hag-2*, the human homologue of the *X. laevis* CG gene *xag-2*, is coexpressed with the estrogen receptor (ER) in breast cancer cell lines. Homeobox gene *Xenopus otx-2* (*Xotx-2*) has been isolated by several groups [15, 16]. A homologue of mouse *otx-2*, it directly controls CG formation and differentiation and spatial and temporal modulation of *Xotx-2* activity limits CG formation at the anterior of the embryo. *otx-2* is expressed in an anterodorsal domain including the presumptive forebrain and CG primordium. Evidence suggests that *otx-2* induce *xcg* activation directly by binding to the xcg promoter to activate its expression. *otx-2* gene, defining an autoregulatory loop. *otx-2* autoactivating is blocked by retinoic acid[17]. *pitx-1*: bicoid-related vertebrate homeobox gene isolated by Hollemann and Pieler [18], involved in patterning anterior structures, including the CG. Experiments show that the *Drosophila orthodenticle* (*otd*) gene, like *Xotx-2*, is able to activate *XAG* during CG morphogenesis in *Xenopus* [19].

Noggin, follistatin, hedgehog, and chordin are found to be inducing factors for cement gland formation [20-22]. Retinoic acid, embryonic fibroblast growth factor (eFGF), bone morphogenetic protein-4 (BMP-4), and Xwnt-8 and involved in CG suppression [23-26].

Direct developing frog *Eleutherodactylus coqui* does not possess a cement gland. Fang and Elinson [8] cloned the *E. coqui* homologue of *otx2*, *Ecotx2* and found that *Ecotx2* protein differs by a single amino acid from *Xenopus* Otx2 protein. Anterior expansion of *otx2* expression in *X. laevis*, which is not found in this expansion in *E. coqui,* is correlated with loss of the cement gland. *Ecotx2* was able to activate expression of the cement gland specific genes xcg and *xag1* when it was misexpressed in *X. laevis* ectoderm. The results indicate that the events which lead to the elimination of cement gland formation in *E. coqui* development are related to the failure of *otx-2* expression in the prospective cement gland primordium. In the species examined here, *X. laevis*: positive control- all four genes (*xcg-1, xag-1, otx-2* and *pitx-1*) are known to occur in this species. *Leptodactylus fuscus* and *Physalaemus pustulosus*, two members of the sub-family Leptodactylidae, one of which has a CG (*P. pustulosus*) and the other of which lacks a CG (*L. fuscus*).

Phyllomedusa trinitatis, a member of the family Hylidae. In most hylids, a CG is present and close homologues of *xcg* and *xag* have been detected [27] in one species tested (*Hyla intermedia*), however, the CG is absent in *P. trinitatis.*

For those species studied here (*Phyllomedusa trinitatis* and *Leptodactylus fuscus*) in which the cement gland is missing, it is of considerable interest to test whether the loss of development of the cement gland in these species occurs by the same developmental pathway changes as in *E. coqui*, or by different routes.

2. Materials and methods

2.1. SEM studies

Photographs at low magnification were taken from the ventral side of the head with focus on the location where the cement gland is expected to be seen at different stages of development in four

species, *X. laevis, P. pustulosus, L. fuscus* and *P. trinitatis*.

2.2. Semi-thin sections

Semi-thin sections (1um) were made for the same species at the appropriate part of the CG or the location where the CG were not apparently present. In the latter case the semi-thin sections were made a few stages just around that in which CG are fully developed in other anuran species.

2.3. Purification of plasmid DNA

Plasmid DNA was prepared with QIAgen prep Miniprep kits according to the manufacturer's protocol.

2.4. Genomic DNA purification

The live tadpoles of four species of anurans, *Xenopus laevis* (late stage) *Leptodactylus fuscus* (metamorphosed) *Phyllomedusa trinitatis* (late stage) and *Physalaemus pustulosus* (middle stage) were killed by transferring straight to liquid nitrogen. The bodies were immediately cut into small pieces and added to digestion tissue solution (700 µl DNA extraction solution + 50 µl proteinase K), then incubated at 55°C with gentle agitation overnight. After spinning down in a micro centrifuge (5 mins 14,000xg), the supernatant was transferred to a pre-spun PLG I heavy tube (20 secs 14,000xg). In the next step, 500 µl 2FC and 500 µl chloroform-Isomyl alcohol were added separately to the solution. In each stage, the solutions were mixed by gentle inversion and were centrifuged for 5 min. at 14,000xg. Aqueous phases were transferred to an eppendorf tube and 100 µl of NH_4OAc was added. The precipitation of DNA was carried out using 100% (v/v) ice cold ethanol and by spinning for 2 min. at 10,000xg. The supernatant was decanted and the DNA pellets were washed with 70% ethanol. The precipitated DNA was placed under the fume hood for 15 min. to remove residual ethanol. 1x TE pH 8.0 in appropriate volume was added to the samples.

2.5. DNA restriction, electrophoresis and southern blothing

Genomic DNA for each sample was digested using appropriate restriction enzymes and buffers. The samples were separated by agarose gel (0.8%) electerophoresis in TAE buffer (57.1 ml glacial acetic acid, 37.2 g $Na_2EDTA.2H_2O$, 242 g Tris base, pH 8.5).

After electrophoresis, the trimmed gel containing DNA was treated with DNA denaturing solution (1.5 M NaCl, 0.5 M NaOH). The DNA was transferred to the membrane by electroblotting for 3hrs at 75V/1500mA as described by Wilson *et al.* [28]. After blotting, the membrane was baked at 80 °C for 1hr, UV crosslinked, and then stored at room temperature until hybridization.

2.6. Probe preparation

Potx-2 was digested with BamHI and XhoI to generate a 0.7 kb probe fragment; pitx and pxcg-1 (reference) were digested with EcoRI to generate 0.53 kb and 0.6 kb fragments (respectively) and pxag-1 was digested with EcoRI and NotI to generate a 2.0 kb probe fragment. These fragments were used to generate labelled probes using plasmids with cloned partial or whole cDNAs of *X. laevis* genes, *otx-2*, *xcg-1* and *xag-1* that were used to make gene specific probes.

^{32}P labelled dCTP (3000 ci/mmol), the primer-It II kit (Stratagene) was used for probe preparation. NucTrap (Stratagene) probe purification column and STE buffer (1 mM EDTA, 10 mM NaCl, 10 mM Tris. Cl pH 7.5, according to the Stratagene prime-It II random primer labelling kit) were used to purify the radiolabelled probe as described by Wilson et al., [28]. The blots were then washed (first round) 4x15 mins in 2x SSC, 0.1% (w/v) SDS at room temperature on a shaking platform. In the second round wash of the blots, the conditions of stringency were adjusted by increasing the temperatures and decreasing the salt concentration.

2.7. Hybridization

Before hybridisation, the blots were prehybridised by adding 10 ml warmed Church buffer in hybridisation tubes. The tubes were put into the oven and rotated for 2-3 hrs at 50-60 °C. The purified probes were denatured at 100 °C for 5 min and were then added directly to the hybridisation tubes containing prehybridised membranes and rotated at 50-60 °C overnight.

3. Results

Figure 1 shows SEM images for the four species. Two round separated cement glands appearing in *Physalaemus pustulosus* and *Xenopus laevis* showed one single sucker shaped CG. The two other species, *Leptodactylus fuscus* and *Phyllomedusa trinitatis* showed no specific CG on the surface. The two prominences appearing on the ventral side of the head in the late two species were first thought to be residual CG or hidden CG from the surface, but semi-thin sections (not shown here) revealed that there was no sign of typical columnar mucus secreting cells in the sections. The tissue in

thick sections also showed no positive reaction to the Alcian Blue staining, as mucus cells are normally stained reddish by this staining technique.

Genomic DNAs were prepared from *X. laevis, L. fuscus, P. trinitatis* and *P. pustulosus* and digested with EcoRI. The DNAs were southern blotted and replicate blots were probed with a sequence from the *Xenopus* genes *xag-1, xcg-1, otx-2* and *pitx-1* (Fig. 2).

Using a stringency: (hybridization 57 °C and washing slight temperature and [salt]).

The *xag-1* probe hybridized to bands of approximately 6.0 kb and 3.5 kb in all species including *Mus musculus*, demonstrating high conservation across species. In *P. trinitatis* a potential additional band at 3.0 kb might indicate a polymorphism present in this sample (Fig. 2). The *xcg-1* probe showed hybridization across all species including *Mus musculus* to two bands of approximately 6.0 kb and 3.5 kb at a stringency of (hybridization 57 °C, wash: 0.5 x SSC, 0.1 % SDS 65 °C).

The *otx-2* probe hybridised to stringency of 6.0 kb and 3.5 kb in *X. laevis, L. fuscus* and *P. pustulosus* but was inconclusive for *Mus musculus* and *P. trinitatis*. In *X. laevis* a potential additional band at 3 kb is indicated (Fig. 2). The *pitx-1* probe did not produce a clear hybridisation signal in any sample (not shown).

4. Discussion

At the morphological study, no evidence of cement gland (CG) in *L. fuscus* and *P. trinitatis* was found. The range of the developmental stages was chosen for histological studies. It was about the time when the CG is normally developing, which is in the middle of the developmental stages. Although we suggest lack of CG for those species, this organ may appear at earlier stages. Because the structure of the eggs at the early stages of development has huge yolk material, sectioning these eggs and gaining the proper tissue was very time consuming with low expectations.

Previous work has shown that a rather large number of genes have a role in CG development in *Xenopus laevis*. Our aim was to test whether some of the same genes are present in other anuran species, especially in those where the CG does not form. When an organ is deleted (missed) from development over evolutionary time, this may occur by deletion of some or all of the genes concerned or by suppression of their expression (more likely they evolve to different functions).

xag-1 and *xcg-1* are clearly conserved across species from frog to mouse and therefore are likely to have a developmental role that is completely independent of amphibian cement gland despite a

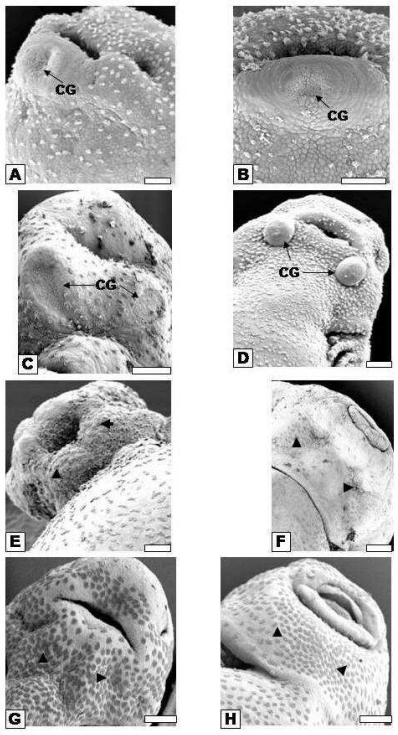

Fig. 1. Cement gland in four species visualized by low resolution (A and B: *Xenopus laevis*, Gosner stages 22 &23; C and D: *Physalaemus pustulosus*, stages 18 & 22; E and F: *Phyllomedusa trinitatis*, stages 20 & 23; *Leptodactylus fuscus*, stages 20 & 23). Arrow or arrow head indicates ventral bulges at locations where CG occurs in other species.
CG= cement gland

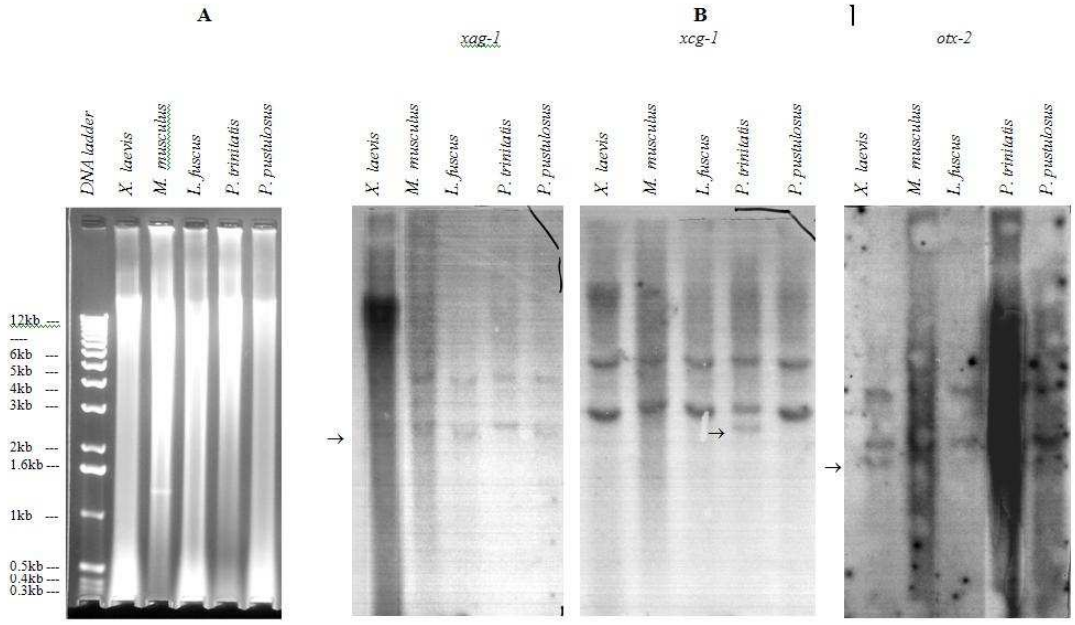

Fig. 2. Genomic DNAs from 1-5 [1) *X. laevis* (positive control), 2) *M. musculus* (negative control), 3) *Leptodactylus fuscus*, 4) *Phyllomedusa trinitatis* and 5) *Physalaemus pustulosus*] were digested with EcoR1 and electrophoresed through 1% agarose (A). Replicate gels were southern blotted and approved (B) with gene sequences from *xcg-1*, *xag-1* and *otx-2* as indicated. Size markers are shown. The extra identified bands are shown/indicated by an arrow

very restricted expression of *xcg-1* in *X. laevis*. *Otx-2* is conserved in the species *L. fuscus* and *P. pustulosus*.

In the only interspecific study of the genes involved in CG development, Pennati et al. [27] detected *xcg-1* and *xag-1* presence and expression in 5 of 6 species studied *(X. laevis* as control; two *Bufo* species; two *Rana* species and one *Hyla*; the exception was *Discoglossus sardus*). However, this is not unexpected as all of these species develop a CG. The results in this study are the first for a species with no CG, even for the genes in the mouse.

At this stage, it can be concluded that, in general the missing cement gland for those species studied here is not because of a lack of the genes responsible for the gland development.

In order to carry this work further, *in situ* hybridiation should be used to determine the actual expression patterns of these genes.

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References

[1] Bennemann, R. & Pietzsch-Rohrschneider, I. (1978). The morphology of the cement gland apparatus of larval *Pterophyllum scalare Cuv. & Val.* (*Cichlidae*, teleostei). *Cell and Tissue Research, 193*, 491-501.

[2] Pottin, K., Hyacinthe, C. & Retaux, S. (2010). Conservation, development, and function of a cement gland-like structure in the fish *Astyanax mexicanus*. *Proceedings of the National Academy of Sciences of the United States of America, 107*(40), 17256-17261.

[3] Pennati, R., Sotgia, C. & De Bernardi, F. (1997). Molecular similarity between ascidian adhesive papillae and amphibian cement gland. *Animal Biology, 7*, 97.

[4] Simeone, A., Acampora, D., Mallamaci, A., Stornaiuolo, A., D'Apice, M., Nigro, V. & Boncinelli, E. (1993). A vertebrate gene related to orthodenticle contains a homeodomain of the bicoid class and demarcates anterior neurectoderm in the gastrulating mouse embryo. *European Molecular Biology Organization Journal, 12*, 2735-2747.

[5] Sive, H. & Bradley, L. (1996). A sticky problem: the *Xenopus* cement gland as a paradigm for anteroposterior patterning. *Developmental Dynamics*, *205*, 265-280.

[6] Nokhbatolfoghahai, M. & Downie, J. R. (2005) Larval cement gland of frogs: comparative

development and morphology. *Journal of Morphology, 263*, 270-283.

[7] Elinson, R. P. (1990). Direct development in frogs: Wiping the recapitulationist slate clean. *Seminars in Cell & Devevelopmental Biology, 1*, 263-270.

[8] Fang, H. & Elinson, R. P. (1999). Evolutionary alteration in anterior patterning: otx2 expression in the direct developing frog *Eleuthrodactylus coqui*. *Developmental Biology, 205*, 233-239.

[9] Downie, J. R. & Nokhbatolfoghahai, M. (2006) Presence and absence of the cement gland in foam-nesting leptodactylids (Anura: Leptodactylidae): implications for the transition to terrestrial development. *Herpetological Journal, 16*, 77-81.

[10] Sive, H., Hattori, K. & Weintraub, H. (1989). Progressive determination during formation of the anteroposterior axis in *Xenopus laevis*. *Cell, 58*, 171-180.

[11] Drysdale, TH. A. & Elinson, R. P. (1992). Cell-migration and induction in the development of the surface ectodermal pattern of the *Xenopus levies* tadpole. *Developmental Growth & Differerentiation, 34*, 51-59.

[12] Jamrich, M. & Sato, S. (1989). Differential gene expression in the anterior neural plate during gastrulation of *Xenopus laevis*. *Development, 105*, 779-786.

[13] Gosner, K. L. (1960). A simplified table for staging anuran embryos and larvae with notes on identification. *Herpetologica, 16*, 183-190.

[14] Thompson, D. A. & Weigel, R. J. (1998). hAG-2, the human homologue of the *Xenopus laevis* cement gland gene XAG-2, is coexpressed with estrogen receptor in breast cancer cell lines. *Biochemical & Biophysical Research Communications, 251*, 111-116.

[15] Blitz , I. & Cho, K. (1995). Anterior neuroectoderm is progressively induced during gastrulation: the role of *Xenopus* homeobox gene *orthodenticle*. *Development, 121*, 993-1004.

[16] Pannese, M., Polo, C., Andreazzoli, M., Vignali. R., Kablar, B., Barsacchi, G., & Boncinelli, E. (1995). the *Xenopus* homologue of *Otx2* is a maternal homeobox gene that demarcates and specifies anterior body regions. *Development, 121*, 707-720.

[17] Gammill, L. S. & Sive, H. (1997). Identification of *otx2* target genes and restrictions in ectodermal competence during *Xenopus* cement gland formation. *Development, 124*, 471-481.

[18] Hollemann, T. & Pieler, T. (1999). *xpitx-1*: a homeobox gene expressed during pituitary and cement gland formation of *Xenopus* embryos. *Mechanisms of Development, 88*, 249-252.

[19] Lunardi, A. & Vignali, R. (2006). *Xenopus Xotx2* and *Drosophila otd* share similar activities in anterior patterning of the frog embryo. *Development Genes & Evolution, 216*, 511-521.

[20] Smith, W. C. & Harland, R. M. (1991). Injected Xwnt-8 RNA acts early in *Xenopus* embryos to promote formation of a vegetal dorsalizing center. *Cell, 67*, 735-765.

[21] Hemmati-Brivanlou, A., Kelly, O. G., & Melton, D. A. (1994). Folistatin, an antagonist of activin, is expressed in the Spemann organizer and displays direct neuralizing activity. *Cell, 77*, 383-95.

[22] Sasai, Y., Lu, B., Steinbesser, H., Geissert, D., Gont, L. K., & De Robertis, E. M. (1994). *Xenopus* chordin: a novel dorsalizing factor activated by organizer-specific homeobox genes. *Cell, 79*, 779-790.

[23] Kolm, P. J. & Sive, H. L. (1995). Regulation of the *Xenopus labial* homeodomain genes, HoxA1 and HoxD1: activation by retinoids and peptide growth factors. *Developmental Biology, 167*, 34-39.

[24] Issacs, H. V., Pownall, M. E. & Slack, J. M. W. (1994). eFGF regulates Xbra expression during *Xenopus* gastrulation. *European Molecular Biology Organization Journal, 13*, 4469-4481.

[25] Fainsod, A., Steinbeisser, H. & De Robertis, E. M. (1994). On the function of BMP-4 in patterning the marginal zone of the *Xenopus* embryo. *European Molecular Biology Organization Journal, 13*, 5015-5025.

[26] Christian, J. L. & Moon, R. T. (1993). Interactions between *Xwnt-8* and Spemann organizer signalling pathways generate dorsoventral pattern in the embryonic mesoderm of *Xenopus*. *Genes & Development, 7*, 13-28.

[27] Pennati, R., Bolzern, A. M., Groppelli, S., Sotgia, C. & Bernardi, F. D. (2000). The adhesive organs of anura: a histological and molecular study. Ital. *Journal of Zoology, 67*, 1-8.

[28] Wilson, J. B., Weinberg, W., Johnson, R., Yuspa, S. & Levine, A. J. (1990). Expression of the BNLF-1 oncogene of Epstein-Barr virus in the skin of transgenic mice induces hyperplasia and aberrant expression of keratin 6. *Cell, 61*, 1315-1327.