

The origins of primitive blood in *Xenopus*: implications for axial patterning

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SUMMARY

The marginal zone in *Xenopus laevis* is proposed to be patterned with dorsal mesoderm situated near the upper blastoporal lip and ventral mesoderm near the lower blastoporal lip. We determined the origins of the ventralmost mesoderm, primitive blood, and show it arises from all vegetal blastomeres at the 32-cell stage, including blastomere C1, a progenitor of Spemann's organizer. This demonstrates that cells located at the upper blastoporal lip become ventral mesoderm, not solely dorsal mesoderm as previously believed. Reassessment of extant fate maps shows dorsal mesoderm and dorsal endoderm descend from the animal region of the marginal zone, whereas

ventral mesoderm descends from the vegetal region of the marginal zone, and ventral endoderm descends from cells located vegetal of the bottle cells. Thus, the orientation of the dorsal-ventral axis of the mesoderm and endoderm is rotated 90° from its current portrayal in fate maps. This reassessment leads us to propose revisions in the nomenclature of the marginal zone and the orientation of the axes in pre-gastrula *Xenopus* embryos.

Key words: Axial patterning, Blood, Fate map, Somite, *Xenopus laevis*

INTRODUCTION

Comparison of the extant fate maps for *Xenopus laevis* reveals a critical inconsistency concerning the marginal zone. Mesoderm arises from the marginal zone, an equatorial torus of cells believed to form mesoderm as a result of inductive interactions between animal and vegetal hemisphere cells (Nieuwkoop, 1969). By convention, the 'dorsal blastoporal lip' forms initially, in the dorsal marginal zone, and the 'ventral lip' forms last, on the opposite side, in the ventral marginal zone (Morgan, 1897). The summary diagrams for fate maps generated by Nakamura and Kishiyama (1971), Keller (1976), and Dale and Slack (data in 1987a, diagram in 1987b) reinforce a belief that the circumference of the marginal zone represents the dorsal-ventral axis of the mesoderm (Morgan, 1897). These maps depict notochord in the dorsal marginal zone, somites lateral to the notochord, and lateral plate mesoderm in the ventral marginal zone. However, there are several important differences between the maps. Keller's original map specifically depicts head mesoderm on the dorsal side, and somites tapering anteriorly and ventrally from the lateral marginal zone to the ventral midline. Dale and Slack specifically place blood in the ventral marginal zone (Fig. 1A). Despite these differences, the maps have been merged into a single image in which all are superimposed on the boundary lines of the original Keller map, and most investigators use this composite map (Fig. 1B). Later studies on morphogenesis (Keller et al., 1989) precipitated major revisions in Keller's map (Keller, 1991), including the depiction of significant amounts of somitic mesoderm extending through the ventral

marginal zone (Fig. 1C). This map is incompatible with the three-signal hypothesis of mesoderm induction (Dale and Slack, 1987b; Smith, 1989), which contends somitic mesoderm occupies the lateral marginal zone and ventral mesoderm the ventral marginal zone. Keller's revised map challenges our understanding of axial patterning in *Xenopus*.

The discrepancy in the maps arises in part because the maps terminate before blood and tail somites differentiate. Nakamura and Kishiyama's map and Keller's original map summarize development to stage 22 (early tailbud stage), while Dale and Slack's map terminates at stage 30 (tailbud stage; Fig. 1D). At stage 30, only the anterior 25 of the eventual 45-plus pairs of somites are present (Nieuwkoop and Faber, 1967), so the source of the posterior somites is unknown. Although statements pertaining specifically to the blood and blood-forming region were made (Dale and Slack, 1987a,b), no data was presented concerning their cleavage-stage origins. The fate maps compiled by Moody (1987a,b) terminate at stage 32-34, when part of the tail, but still no blood, have formed, and these maps do not define specifically the progenitors of tail somites nor primitive blood. Thus, despite the construction of numerous fate maps, the origins of primitive blood and tail somites are unknown in *Xenopus*.

The tissues in contention are important in considering axial patterning because they provide critical information about the emerging dorsal-ventral and anterior-posterior axes. Primitive blood is the ventralmost mesoderm in the body plan, arising from the ventral blood islands, and the source of the blood islands should be designated the ventral marginal zone. Tail somites are posterior, dorsal mesoderm, and the region from

which they originate should be included in the dorsal marginal zone. If Keller's revised map is correct, and somites arise from the region currently called the ventral marginal zone, then the circumference of the marginal zone does not represent the dorsal-ventral axis, and the region designated the ventral marginal zone is misnamed. Consequently, the orientation of the dorsal-ventral axis in pre-gastrula embryos becomes unknown. The location of the ventral marginal zone can be determined by finding the source of the primitive blood, and once this region is identified, the correct orientation of the dorsal-ventral axis can be deduced.

Most experiments concerning mesodermal patterning in *Xenopus* assume that primitive blood arises from the region currently designated the ventral marginal zone. It is important therefore to establish unequivocally the fates of cells in this region. For this reason, we determined the lineage of posterior somites and primitive blood from the 32-cell stage (stage 6; Fig. 1E) to the tadpole (stage 41; Fig. 1F), when both tissues are present. We find that the region historically called the ventral marginal zone gives rise to posterior somites as well as posterior, ventral blood islands. Blood arises from the vegetal region of essentially all 360° of the marginal zone. A reassessment of extant fate maps constructed from the 32-cell stage leads us to reorient the dorsal-ventral axis of the mesoderm and the endoderm in the marginal zone and thus has significant implications for axial patterning in *Xenopus*.

To minimize confusion in this report, we employ Spemann's terminology to refer to the lips of the blastopore: the region commonly called the 'dorsal lip' is Spemann's 'upper lip', and the region commonly called the 'ventral lip' is Spemann's 'lower lip'. 'Dorsal' and 'ventral' are used in their currently accepted senses throughout this report, until we redefine the dorsal and ventral marginal zones in the Discussion.

MATERIALS AND METHODS

Embryos

Embryos obtained from adult frogs (*Xenopus* 1) by standard methods were dejellied in 2% cysteine HCl, pH 8.0, 15 minutes after fertilization. Nieuwkoop and Faber's staging criteria (1967) and Nakamura and Kishiyama's blastomere nomenclature (1971; Fig. 1E) were used. All experiments were replicated with eggs from three to ten females.

Lineage tracing with rhodamine dextran amine (RDA)

Regularly cleaving embryos in 0.1× MMR (Marc's modified Ringer's) displaying strong dorsal-ventral pigmentation differences were marked at the cleavage plane on the lightly pigmented side at the 4- to 8-cell stage with Nile blue sulfate. Marks were within 15° of the midline of the upper blastopore lip more than 95% of the time. At the 32-cell stage, embryos with four tiers of eight blastomeres were selected. Embryos with eight equal-sized D blastomeres in contact with the vegetal pole were never observed. We therefore used embryos with large D2 and D3 blastomeres that contacted the vegetal pole, and smaller D1 and D4 blastomeres that did not (see Fig. 1E). The embryos were transferred to 6% ficoll/0.1× MMR and injected by air pressure into appropriate blastomeres with 1 nl of rhodamine-dextran amine (RDA, 33 µg/ml in H₂O; Molecular Probes) at the late 32-cell stage. Embryos were cultured in 0.1× MMR plus 10 µg/ml gentamycin. Specimens were fixed 1 hour in MEMFA (0.1 M MOPS, pH 7.4, 2 mM EGTA, 1 mM MgSO₄, 3.7% formaldehyde) and stored

in 100% methanol. Specimens were cleared in benzyl benzoate:benzyl alcohol (2:1 v/v) for observation.

We confirmed our technique by injecting individually, blastomeres A1, B1, C1, D1, A4, B4, C4, D4 or C3 with RDA and culturing embryos to stage 30 or 41. We observed no major discrepancies from the results of Dale and Slack (1987) nor Moody (1987b) other than those described in the text. At stage 41, fluorescent cells circulated through the vasculature in embryos injected into C1, D1, C3, C4 or D4 blastomeres. The natural red autofluorescence of the embryo precluded routine photographic documentation of circulating cells in live embryos, so we switched to injection of mRNA encoding the green fluorescent protein as a lineage tracer.

Lineage tracing with green fluorescent protein

1 nl (0.5-1.0 µg/ml) of an in vitro synthesized, capped mRNA (Message Machine) encoding a modified GFP (GFP.RN3; Zernicka-Goetz et al., 1996) was injected into identified blastomeres at the 32-cell stage, or 0.5 nl at the 64-cell stage. Embryos were cultured to early gastrula, and screened on a dissecting microscope under combined epifluorescence and epi-illumination to ensure that appropriate blastomeres had been labeled (using the mapping data of Vodicka and Gerhart, 1995). Screening eliminates incorrect blastomere injections caused by fallacious identification of the dorsal side at the 4-8 cell stage.

Live embryos at stage 41 were examined for circulating green blood and green somitic cells under epifluorescence illumination with an FITC filter. Circulating blood is readily observed at several locations in the embryo at stage 41, including the gill arches, heart, retina and major blood vessels in the tail. Multiple vessels were observed in every embryo. A single cell per embryo is detectable and scored as positive; however, all positive embryos in Table 2, except those injected into blastomere B1, had numerous GFP-positive, circulating cells.

As our major goal was to determine the origins of primitive blood, which is best done in living embryos using the criterion of circulation through the vasculature, we mapped with GFP, which has distinct advantages and disadvantages. GFP is non-toxic, does not bleach and is easily observed by conventional epifluorescence microscopy. Moreover, the signal generally increases with time during early developmental stages, and is present at stage 41 (Zernicka-Goetz et al., 1996). The major disadvantage of GFP is that it is not amenable to standard histological techniques such as paraffin sectioning, which precludes making volume measurements. However, blood is not particularly suited to volume measurements. Finally, it takes several hours for detectable levels of GFP protein to be synthesized, so one cannot assess immediately whether injected message spreads uniformly throughout a blastomere.

Fate mapping was carried out by frequency distribution. The numbers given in Tables 1 and 2 are the percentages of surviving, stage 41 embryos with labeled cells in the specified tissue after injection into a specific blastomere at stage 6. Mapping by frequency distribution does not permit conclusions about volume or mass contributions from specific blastomeres, but does identify which blastomeres routinely contribute to a tissue. Given that injected mRNAs are non-uniformly distributed in the cytoplasm and therefore not inherited by all progeny, quantitative analysis based on message injection represents underestimates in almost all circumstances. As our goal was to identify the cleavage-stage origins of primitive blood, we settled for the information generated by frequency distribution mapping.

Embryos were photographed under epifluorescent illumination or under combined epifluorescent and epi-illumination with fiber optic lighting, using Kodak EKT 160 slide film and a 35 mm camera. Montages were assembled in Adobe Photoshop or Canvas. Fig. 3A was constructed by superimposing three fluorescent, time-lapse images of a C1-injected, stage 39 tadpole, captured with a SIT camera, onto a phase image, using Adobe Photoshop. The first fluorescent

image was used for background subtraction, the second pseudo-colored red and the third pseudo-colored green.

Quantitation of blastomere contributions to blood

The tip of the tail was cut and blood collected in a heparinized capillary tube from individual, stage 41 embryos that had been injected at stage 6. Blood was spread on slides with marked wells, and the number of green blood cells counted under epifluorescent illumination. The slides were fixed, stained with Wright's and Geimsa, and random fields of blood cells photographed. The cells per field were counted, and the total cells per well and the percentage of blood cells expressing GFP calculated. To establish that GFP-expressing cells also contain hemoglobin, fields of GFP-positive cells obtained by tail bleed were photographed, then histochemically stained with benzidine for 10 minutes (Hemmati-Brivanlou and Thomsen, 1995) while mounted on the microscope.

Generation of dorso-anteriorized embryos

Eggs from albino females and sperm from pigmented males were used to generate embryos with greatly reduced pigmentation. (Some pigmentation facilitates scoring using the dorsoanterior index of Kao and Elinson, 1988). 32- to 64-cell stage embryos were exposed to 0.3 M LiCl₂ in 1/10 MMR for 11-13 minutes. The embryos were washed extensively in 1/10 MMR (6 changes over 3 hours) and cultured to stage 41. Deuterium oxide (Sigma) treatment followed the protocol described by Scharf et al (1989). In situ hybridization for α T3 globin followed the protocol of Harland (1991).

RESULTS

Tailbud and tadpole fates of the ventral marginal zone progenitors, B4 and C4

Stage 30 tailbud and stage 41 tadpole embryos (Fig. 1D,F) that were injected into blastomere C4 or B4 at stage 6 (32-cell stage, Fig. 1E) with RDA were inspected as intact embryos. At stage 30, labeled B4 and C4 progeny are confined to regions posterior of the head (Fig. 2A,B), including trunk somites, tissue surrounding the proctodeum, and undifferentiated tissue in the tailbud anlagen. Labeled tissue is not found anterior to the otic vesicle, nor in the ventral region immediately posterior to the heart and liver anlagen (Fig. 2A,B). Our observations are in accordance with the schematic, graphical and tabular presentations of Dale and Slack's data for tailbud embryos (see Figs. 5 and 6 and Table 3 for B4 and C4 results in Dale and Slack, 1987a).

At stage 41, when the tailbud anlagen has generated another 20-plus pairs of somites, both B4 and C4 progeny contribute heavily to newly formed somites posterior to the proctodeum (Fig. 2C,D). Again, no label is present immediately posterior of the heart and liver anlagen. This is the anterior region of the ventral blood islands (VBI) that first expresses early blood markers, including α T3 globin and GATA-1 (Kelley et al., 1994, Walmsley et al., 1994), so neither C4 nor B4 form the anterior VBI. By stage 41, blastomere C4 contributes to posterior regions of the VBI (Fig. 2D), whereas B4 does not, and fixed blood cells are seen in the heart of the C4-labeled embryo in Fig. 2D (asterisk), but not in the heart of the B4-labeled embryo (Fig. 2C). Thus, the ventral marginal zone contributes heavily to tail somitic mesoderm at stage 41, and the lower portion of the ventral marginal zone contributes to primitive blood and the VBI, but only to the posterior VBI. The source of the anterior and mid-axial VBI is discussed below.

Origins of the trunk and tail somites

To find all sources of trunk and tail somites, we injected individual blastomeres at stage 6 with an mRNA encoding green fluorescent protein (GFP), and scored live embryos for labeled cells in chevron-shaped somites at stage 41. The results are summarized in Table 1. Trunk and tail somites descend from all B- and C-tier blastomeres. A-tier blastomeres routinely contribute many cells to many somites (see Fig. 3F). D-tier blastomeres make occasional, small contributions (about ten somite cells per blastomere). Our results confirm earlier observations that B- and C-tier blastomeres contribute heavily to somites (Dale and Slack, 1987a; Moody, 1987b), and confirm Moody's observation that somites originate from the A-tier, which usually is not included in the marginal zone.

All stage 6 vegetal hemisphere blastomeres, including those that populate Spemann's organizer, contribute to primitive blood

Using GFP mRNA, we determined the cleavage-stage origins of primitive blood by examining live, stage 41 embryos for circulating, labeled cells. We scored at stage 41 for several reasons. The ventral blood islands form in an anterior-to-posterior (A-P) progression (Kelley et al., 1994), with the first cells circulating at stage 35. The VBI begins to disintegrate around stage 40, also in an A-P progression. Scoring at stage

Table 1. Stage 6 origins of stage 41 trunk and tail somites

| Blastomere | 4 | 3 | 2 | 1 |
|------------|----------------------|---------------------|----------------------|----------------------|
| A | 89% <i>n</i> =35 | 60% <i>n</i> =10 | 77% <i>n</i> =13 | 43% <i>n</i> =14 |
| B | 100% <i>n</i> =29 | 100% <i>n</i> =4 | 100% <i>n</i> =6 | 100% <i>n</i> =22 |
| C | 100% <i>n</i> =34 | 100% <i>n</i> =9 | 100% <i>n</i> =19 | 97% <i>n</i> =34 |
| D | 27%* <i>n</i> =22 | 8%* <i>n</i> =25 | 13%* <i>n</i> =8 | 32%* <i>n</i> =19 |

The distribution of prospective somitic tissue, determined by frequency distribution mapping. *n*, the number of normal, surviving, stage 41 embryos following injection at stage 6 with mRNA-GFP. The percentage of survivors expressing GFP in somitic cells for each blastomere is shown. The anteriormost 6-7 somites in *Xenopus*, which are not chevron-shaped, are not included in the data. *Indicates only a few cells (approx. 10) were labeled.

Table 2. Stage 6 origins of stage 41 primitive blood

| Blastomere | 4 | 3 | 2 | 1 |
|------------|---------------------|---------------------|---------------------|---------------------|
| A | 19% <i>n</i> =32 | 12% <i>n</i> =16 | 0% <i>n</i> =16 | 0% <i>n</i> =32 |
| B | 0% <i>n</i> =19 | 0% <i>n</i> =10 | 0% <i>n</i> =11 | 9%* <i>n</i> =22 |
| C | 81% <i>n</i> =27 | 86% <i>n</i> =14 | 95% <i>n</i> =22 | 98% <i>n</i> =40 |
| D | 65% <i>n</i> =31 | 80% <i>n</i> =20 | 67% <i>n</i> =12 | 73% <i>n</i> =22 |

The distribution of prospective primitive blood, determined by frequency distribution mapping, using the criterion of circulation. *n*, the number of normal, surviving, stage 41 embryos following injection at stage 6 with mRNA-GFP. The percentage of survivors expressing GFP in circulating cells for each blastomere is shown. Individual blastomeres were mapped using eggs from four or more females. *Blastomeres scored positive which had ten or fewer labeled cells in circulation. All other blastomeres scored positive gave rise to numerous labeled cells in circulation.

41 should allow adequate time for the full A-P extent of the VBI to contribute to the circulating cell population, but safely allows us to avoid scoring definitive blood, which enters the circulation from a second site of hematopoiesis later in development (Kau and Turpen, 1983).

Individual, GFP-expressing blood cells are readily observed in living embryos under epifluorescent illumination. They fluoresce as brightly as other labeled cell types and circulate

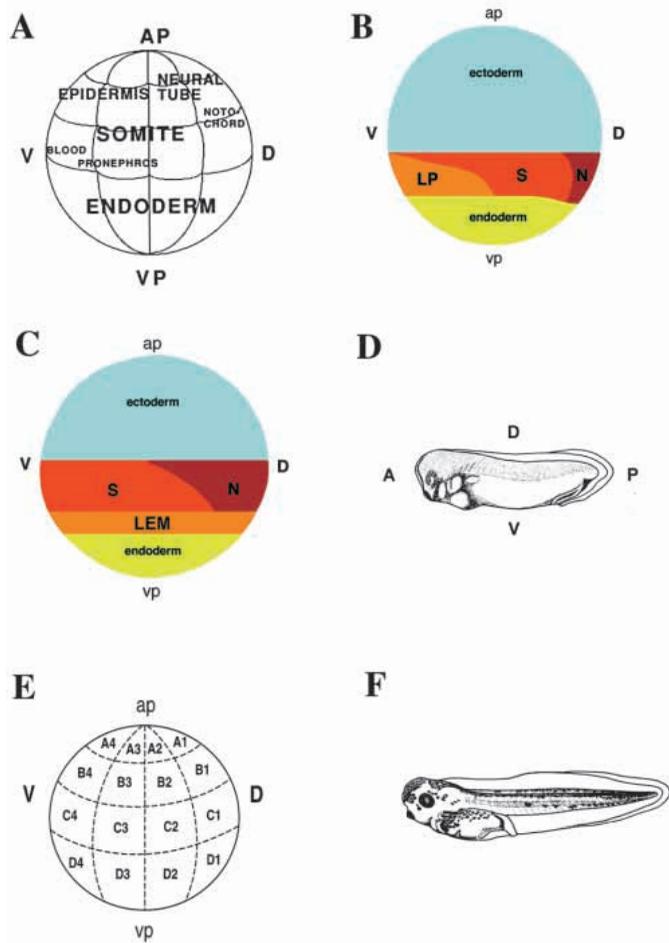


Fig. 1. Summary tailbud fate maps of the marginal zone and pertinent Nieuwkoop/Faber (N/F) stages for *Xenopus laevis*. (A) The summary fate map for stage 6-30 of Dale and Slack (1987a,b) taken from Smith (1989). (B) The composite fate map of the marginal zone. In the ventral marginal zone, a small amount of somitic mesoderm is present, while lateral plate mesoderm, which includes blood, predominates. This composite map, shown in textbooks, review articles and seminars is an amalgamation of many fate maps superimposed onto the boundary lines of the original fate map (stage 10-22) compiled by Keller (1976). See text for further description. (C) Keller's revised map (1991). The ventral marginal zone includes significant prospective somitic tissue (shown in red on the ventral side), while leading edge mesoderm (orange), which includes lateral plate, intermediate and head mesoderms, resides in the vegetal portion of the marginal zone. (D) N/F stage 30 tailbud. (E) N/F stage 6 (32-cell stage), with the blastomere nomenclature of Nakamura and Kishiyama (1971) indicated. (F) N/F stage 41 tadpole. A, anterior; ap, animal pole; vp, vegetal pole; D, dorsal; LEM, leading edge mesoderm; LP, lateral plate; N, notochord; P, posterior; S, somites; V, ventral.

through the vasculature. Time-lapse images, captured with a lowlight level, SIT camera and superimposed in Fig. 3A, show individual blood cells that move a short distance between exposures. To capture images using conventional fluorescence microscopy and photography, many labeled cells must circulate and long exposure times are required. The resulting images show arteries and veins fluorescing faintly in comparison to labeled, immobile tissues, and discrete blood cells are not distinguishable (below, Fig. 3C). Fig. 3A demonstrates that the weak fluorescence of the vasculature in long exposures is caused by the accumulated traces of circulating cells, and are not lineage-labeled endothelial cells.

Observations on live embryos are summarized in Table 2. Progeny from all vegetal hemisphere blastomeres at stage 6 (i.e., C- and D-tiers), and occasionally a few blastomeres in the animal hemisphere (notably A4 and A3) form primitive blood at stage 41. B-tier blastomeres make minor contributions only very rarely. As examples, we show typical embryos labeled in C1 or B1 (Fig. 3B,D). Progeny of these blastomeres populate Spemann's organizer (Vodicka and Gerhart, 1995). Both

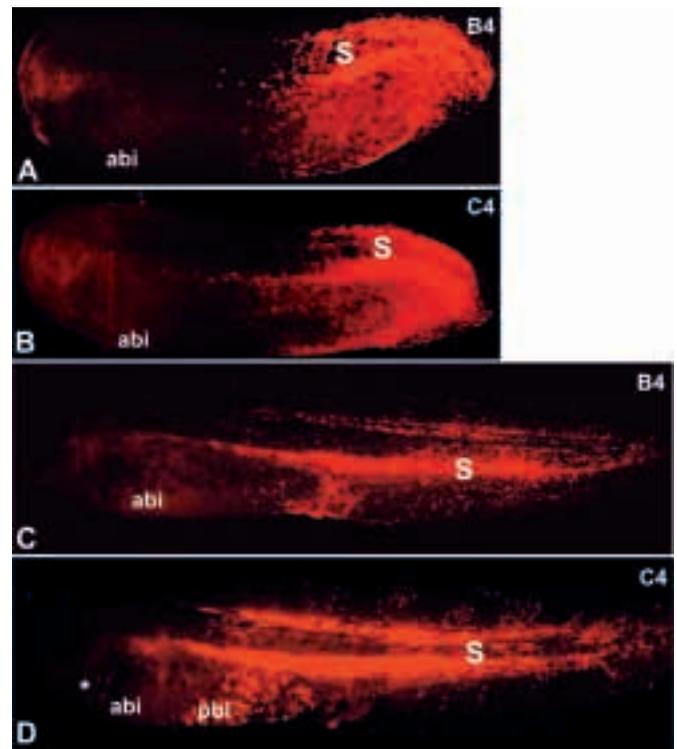


Fig. 2. Tailbud and tadpole stage, whole-mount embryos, labeled with RDA at stage 6, photographed under combined epifluorescence and epi-illumination to reveal labeled and unlabeled tissue. Dorsal is up and anterior to the left in all images. Faint red is the embryo; bright red is RDA label. (A,B) B4- and C4-labeled, stage 28-30 embryos. No label is present in the head. Trunk somites (s), lateral plate and the tailbud anlagen are heavily labeled. The region including the anterior VBI (abi) is unlabeled. (C) B4-labeled, stage 41 tadpole. Trunk/tail somites, tail epidermis and the proctodeum are heavily labeled. The anterior VBI (abi) is unlabeled. (D) C4-labeled, stage 41 embryo. Trunk/tail somites and epidermis are labeled. The region including the posterior VBI (pbi) and posterior lateral plate is labeled. Labeled blood cells are fixed in the heart (asterisk) of the C4-injected embryo.

blastomeres give rise to the tissues previously identified: C1 progeny populate the notochord, head and heart mesoderms, somitic mesoderm and pharyngeal endoderm; B1 progeny populate the notochord, the neural tube including much of the brain and the floorplate, and somitic mesoderm (Fig. 3B,D). In addition, GFP-expressing cells are found circulating throughout the vasculature in C1-injected tadpoles (Fig. 3A,C), but not in B1-injected embryos (Fig. 3E). Examination of the ventral midline region reveals the critical difference between B1- and C1-injected embryos. Few, if any, B1 progeny populate the VBI region (arrow, Fig. 3D), while C1 progeny always occupy the ventral midline (arrows, Fig. 3B). A similar pattern is observed for all B- and C-tier progeny (Fig. 2C,D). D-tier blastomeres make frequent contributions to primitive blood (Table 2), albeit at lower frequencies than C-tier blastomeres, and their progeny populate the ventral midline of the tadpole (not shown).

Current models of amphibian mesoderm induction predict that cells populating Spemann's organizer near the dorsal midline do not form blood. To confirm the results of C1-injection and to further refine the map in the region of Spemann's organizer, we injected the medial daughter of blastomere C1 with GFP mRNA, and scored at stage 41. 83% ($n=23$) of these embryos had labeled blood, indicating that the primitive blood territory approaches within approximately 22.5° of the dorsal midline in the region of the organizer (not shown).

We refined the boundaries of the blood territory in the vegetal hemisphere by mapping the upper and lower daughters of C- (either C1 or C2) and D-tier (either D1 or D2) blastomeres from the 64-cell stage. We observed labeled blood in 9% of upper C blastomeres ($n=11$), 93% of lower C blastomeres ($n=15$), 70% of upper D blastomeres ($n=10$) and 0% of lower D blastomeres ($n=17$). We conclude that, within the vegetal hemisphere, the blood territory occupies the lower portion of the marginal zone, and not the region immediately adjacent to the vegetal pole.

Judging from visual observation of live embryos, only a minor proportion of the blood cells express GFP in any individual, injected embryo, suggesting that no single blastomere serves as a major source of primitive blood at stage 41. To provide some quantification of the contribution of vegetal blastomeres, we collected blood at stage 41 from individuals injected at the 16-cell stage, and determined the percentage of blood cells labeled. The results shown in Table 3 are typical of three

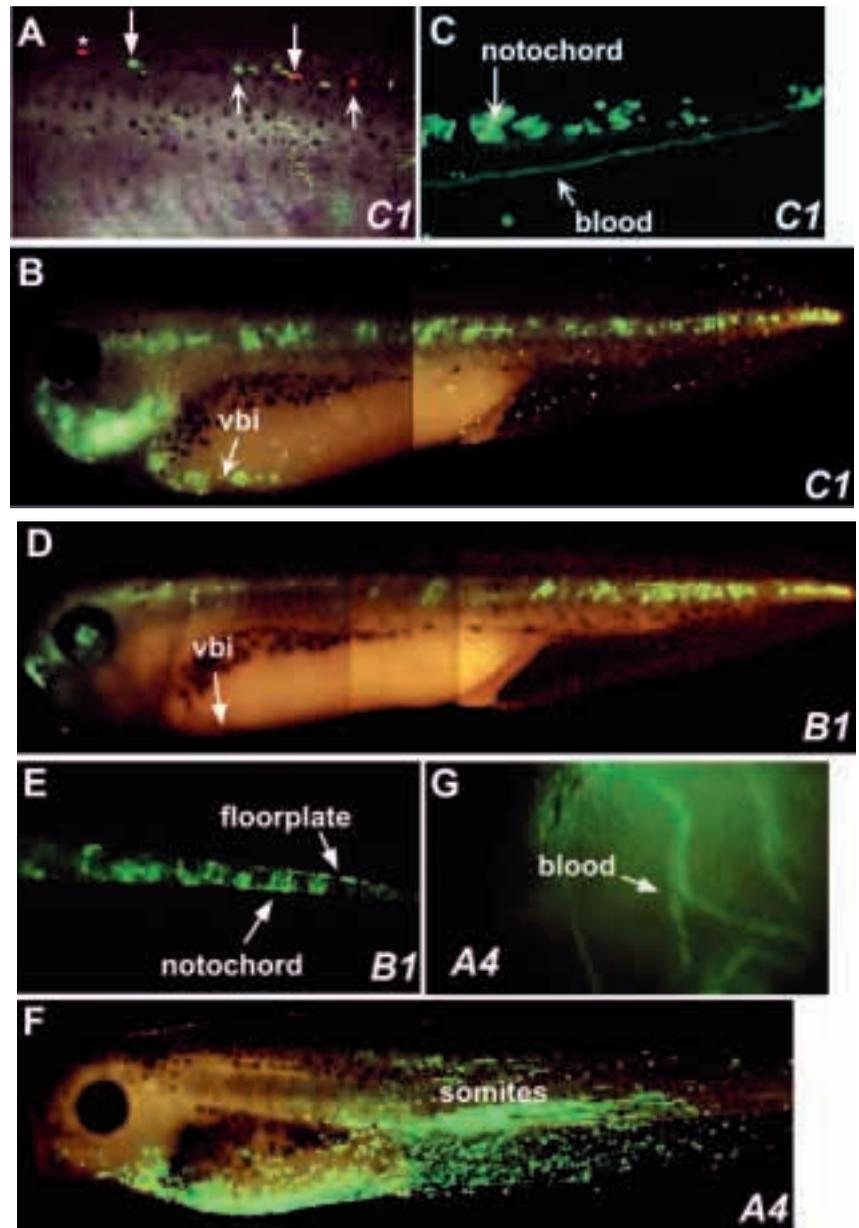


Fig. 3. Primitive blood in stage 39 tadpoles. Dorsal is up and anterior to the left in all images. (A) GFP-expressing blood cells in the tail of a C1-injected embryo. Three fluorescent images captured with a SIT camera, superimposed on a phase image of the embryo. The first fluorescent exposure was used for background subtraction, the second pseudo-colored red and the third pseudo-colored green. The image shows individual, labeled cells circulating in a tail vein. Arrows of different shapes indicate individual cells at the two time points. One labeled blood cell (asterisk) moves out of the field of view between the two time points. (B) C1-injected embryo. Notochord, somites, head mesoderm and the anterior, ventral region (arrow) including the region of the anterior VBI (vbi) are labeled. (C) Tail of C1-injected embryo. GFP-expressing, circulating blood in the caudal artery and vein. (D) B1-injected embryo. Notochord, brain, floorplate and somites are labeled. The VBI region (arrow) and leading edge mesodermal derivatives are unlabeled. (E) Tail of B1-injected embryo. Exposure time is the same as in C. No labeled blood circulates in the vasculature. The notochord and floorplate are labeled. (F) A4-injected embryo. Progeny include tail somites and ventral epidermis. (G) A4-derived, labeled blood circulates in the contralateral gill arches of the embryo shown in F. Combined epifluorescence and epi-illumination is used in images in B, D and F. The images in C, E and G are epifluorescence only.

Table 3. Vegetal blastomere contributions to stage 41 blood

| Individual | Blastomere | | |
|------------|------------|------|------|
| | CD1 | CD2 | CD4 |
| i | 0.0* | 3.7 | 1.3 |
| ii | 1.2 | 4.9 | 2.5 |
| iii | 1.9 | 6.3 | 16.7 |
| iv | 16.5 | 23.7 | 18.3 |

All numbers are percentages of blood cells expressing GFP. Embryos injected into one blastomere at the 16-cell stage were cultured to stage 41. Blood collected by tail bleed was spread in a fixed area on a slide. The number of GFP-expressing cells was counted immediately, and the total number of blood cells was counted after staining with Wright-Geimsa. *A false negative as a result of sampling error. This embryo had GFP-expressing blood cells circulating in the vasculature, but none was collected in the drop from the wound.

trials. CD1 (i.e., the mother blastomere of C1 and D1) contributions range from 0-16.5%, CD2 from 3.7-23.7% and CD4 from 1.3-18.3%. The variability in the range of values reflects the variability in individual embryos as viewed in the microscope – for any given blastomere that routinely contributes to blood, some embryos will be very heavily labeled and some less labeled. The range of values observed for individual vegetal blastomeres are comparable at stage 41. Each vegetal blastomere contributes a proportion of the blood cells at stage 41, and we conclude that primitive blood cells descend from a widespread area of the cleavage-stage embryo.

Animal hemisphere contributions to blood

Animal hemisphere blastomeres contribute to blood at low frequencies. We observed labeled, circulating cells from injections of blastomere A4 (Fig. 3G) or A3, but none from A2- or A1-injected embryos. We observed A-tier-derived blood using eggs from 6 of the 10 females tested. Very small amounts of B1-derived blood formed in 2 of 22 B1-injected embryos. Two labeled blood cells (out of hundreds or thousands of blood cells) were found in one embryo and about ten in the second embryo. We detected no other B-tier contributions. In comparison, labeled blood cells descended from individual C-, D-, and A-tier blastomeres were too numerous to count in live embryos. We conclude that A4 and A3 progeny contribute substantially to primitive blood in some embryos, whereas A2, A1 and all B progeny do not.

Circulating, GFP-positive cells from C1 and A4 are erythrocytes

Our criteria for blood cells in live embryos was circulation through the vasculature. Of the blastomeres identified as sources of primitive blood, the most surprising were C1, a major progenitor of Spemann's organizer, and A4, a major progenitor of the animal cap at early gastrula stage. To test whether circulating, GFP-positive cells are erythrocytes, we collected blood from C1-, A4- or the medial daughter of C1-injected embryos, visualized GFP fluorescence, and stained with benzidine, a histochemical indicator of hemoglobin. Benzidine stains only blood in tadpoles (Fig. 4A; Hemmati-Brivanlou and Thomsen, 1995). In the blood of C1- ($n=6$), A4- ($n=3$), and the medial daughter of C1-injected embryos ($n=4$), 100% of the GFP-labeled cells stain with benzidine, demonstrating that circulating, GFP-labeled cells contain hemoglobin and are erythrocytes (Fig. 4B-D).

Primitive blood forms from vegetal blastomere progeny in an anterior-posterior progression

The ventral blood islands coalesce in a spatial and temporal progression, beginning in the anterior, near the liver diverticulum, and spreading posteriorly towards the proctodeum, during the late neurula and tailbud stages (Kelley et al., 1994). This progression correlates with the spatial distribution of the leading edge mesoderm cells in the ventral midline region (Keller, 1976). CD1 progeny populate the anterior end of the ventral midline region (Fig. 3B), CD2 and CD3 progeny populate the mid-axial, and CD4 progeny populate the posterior end of the ventral midline (Fig. 2D). To determine whether there are spatial and/or temporal progressions in blood formation from vegetal blastomeres, we examined injected embryos from stage 35, when circulation begins, through stage 41, when all blood is still primitive blood derived from the ventral blood islands. Table 4 shows a typical set of results from three trials. GFP-expressing blood is observed first in C1-injected embryos, by stage 36. C2 progeny circulate by stage 37/38 and C3 and C4 progeny by stage 41. Thus, blood cells enter the circulation from the VBI in a spatial and temporal progression that matches the A-P pattern in which leading edge mesoderm cells coalesce into the VBI.

Dorso-anteriorized embryos form primitive blood

The formation of ventral mesodermal tissues such as blood by blastomeres near the dorsal midline, especially C1, contradicts current hypotheses of mesoderm induction in *Xenopus* (Dale and Slack, 1987b and references therein; Heasman, 1997). To confirm independently that primitive blood arises from tissue near the dorsal midline, we examined embryos with modified body plans for globin, a marker of blood. The amphibian body plan can be modulated from extremely ventro-posteriorized to extremely dorso-anteriorized forms by various treatments, including ultraviolet irradiation, and Li^+ - or deuterium oxide-exposure, respectively. These forms are evaluated using the dorso-anterior index (DAI; Kao and Elinson, 1988). UV-generated, DAI 0 embryos form blood but not notochord; it is believed these represent a radialization of ventral midline tissues, and are considered hyperventralized. Li^+ -generated, DAI 10 embryos, believed to represent a radialization of dorsal midline tissues and to be hyperdorso-anteriorized, have abundant notochord, but also blood, as judged by morphological criteria (Cooke and Smith, 1988). We confirm the presence of red blood cells in Li^+ -treated, DAI 10 embryos (not shown). $\alpha T3$ globin, expressed in control stage 40 embryos (Fig. 5A) in the posterior VBI and in cells throughout the vasculature, is expressed in Li^+ -treated, DAI 10 embryos

Table 4. Temporal and spatial progression of primitive blood formation from C-tier blastomeres

| Blastomere | <i>n</i> | stage 36 | stage 37/8 | stage 41 |
|------------|----------|----------|------------|----------|
| C1 | 3 | 3 | 3 | 3 |
| C2 | 4 | 0 | 4 | 4 |
| C3 | 5 | 0 | 1 | 4 |
| C4 | 2 | 0 | 0 | 1 |

n, number of embryos injected into a specific blastomere at stage 6. The number of embryos with GFP-expressing blood at specified stages is shown.

(Fig. 5B). One caveat of this experiment is that Li^+ -exposure after the mid-blastula transition causes ventralization (Yamaguchi and Shinagawa, 1989), and failure to remove all of the Li^+ after early treatment may explain the presence of blood in these embryos.

To circumvent problems interpreting Li^+ -generated, high DAI embryos, we made dorso-anteriorized embryos by exposure to deuterium oxide. A previous report states that D_2O -generated, DAI 9 embryos have no blood in their hearts (Scharf et al., 1989). We confirm blood is absent in the hearts of these embryos, but find it in the yolky half of the embryo (presumably the blood islands), as judged by benzidine staining (not shown) and in situ hybridization for globin (Fig. 5C). Thus, tissue that contributes to primitive blood is present in hyperdorso-anteriorized embryos, and presumably also near the dorsal midline in normal embryos.

DISCUSSION

Revising the fate map

To resolve a critical discrepancy in the extant fate maps, we determined the cleavage-stage origins of primitive blood and posterior somites in *Xenopus laevis*. Our data support Keller's revised fate map (1991) that contends posterior somites occupy much of the region currently called the ventral marginal zone. Both C4 and B4 blastomeres contribute significantly to trunk and tail somites, the proctodeum and tail epidermis. Neither blastomere routinely contributes to the head (i.e., tissues anterior to the otic vesicles, Fig. 2C,D; and Dale and Slack, 1987a). C4 contributes to posterior lateral plate mesoderm, including the posterior VBI; B4 makes negligible contributions to lateral plate and ventral mesoderm. Given that B4 and C4 progeny populate *posterior*, dorsal as well as *posterior*, ventral tissues in the embryo, we suggest that 'posterior marginal zone' more accurately describes this region than 'ventral marginal zone'.

Most importantly, we find the progenitors of primitive blood are not restricted to blastomere C4, as reported by Dale and Slack (1987b). We find that blood arises from all vegetal hemisphere cells at stage 6, and some animal hemisphere cells. Refined mapping at the 64-cell stage reveals that lower C- and upper D-tier progeny contribute to primitive blood, placing the blood territory in the lower portion of the marginal zone. We find that blastomere C1 and the medial daughter of blastomere C1, cells that contribute to Spemann's organizer, routinely form primitive blood. Confirmation of our results for stage 6 comes from examination of Dale and Slack's data (Fig. 5 in 1987a), where the region corresponding to the anterior VBI is labeled by A4, C1, C2 and D1. In addition, a recent report by Tracey et al. (1998) demonstrates that the anterior end of the ventral blood islands originates from the dorsal side of the embryo, and that dorsal marginal zone explants express *Xaml-1*, a marker of several lineages including hematopoietic stem cells. The results of Tracey et al. (1998) support our contention that the region currently called the 'dorsal marginal zone' contains cells fated to form ventralmost mesoderm.

A summary fate map for the mesoderm including our modifications is shown in Fig. 6A. (In light of the fact that we have not mapped all tissues at stage 41, we caution that this

diagram is provisional. The distribution of mesodermal tissues other than somites and blood is based on the results of Moody (1987b), for reasons discussed below. A true stage 6-41 map is currently under construction.) Prospective somitic mesoderm occupies a large portion of the ventral marginal zone. Primitive blood forms from tissue along the vegetal boundary of the marginal zone with the endoderm, the region that forms leading edge mesoderm during gastrulation. As fate mapping the medial daughter of C1 from the 64-cell stage limits resolution to 22.5° sectors, we cannot determine at present if the blood territory reaches the dorsal midline, or only comes within 22.5° of the median. Expression of αT3 globin in highly dorso-anteriorized embryos (Fig. 5B,C) suggests the blood territory lies at least as close to the midline as the heart. For simplicity, we show blood through 360° of the vegetal marginal zone.

We are unable to provide accurate estimates of the percentage of blood cells contributed by specific blastomeres. Due to several unforeseen limitations of our experiments, the strict criteria required for determination of mass contributions are not met. First, lineage tracer must be inherited uniformly by all progeny. mRNAs injected into *Xenopus* embryos are not believed to be homogeneously inherited. Indeed, screening at the early gastrula stage revealed that labeled clones often are not uniformly labeled. Second, progeny must synthesize detectable levels of protein through to stage 41. Zernicka-Goetz et al. (1996) concluded that GFP.RN3 mRNA injected at stage 2 begins to fall by stage 18 and is at 0.5% of its initial level by stage 46, so message levels and synthesized protein are likely decreased by stage 41. During repeated monitoring of individual embryos with labeled blood from stage 41-45, we observed that the fluorescence intensity of labeled blood cells often decreased. This may reflect decreasing protein levels, or it may reflect specific fluorescence quenching by hemoglobin in maturing erythrocytes. Both the excitation and emission spectra of various GFPs (Heim and Tsien, 1995) overlap with peaks in the absorption spectra of hemoglobin (Lemburg and Legge, 1949). This means the constant amount of light emitted by the source on the microscope excites fewer GFP molecules in cells filled with hemoglobin, reducing potential signal generation, and actual signal generated (GFP-emitted light) is lost, again due to absorption by hemoglobin. Quenching will not be linear in *Xenopus* embryos. As blood is produced in a spatial and temporal gradient in the embryo (Table 4), progeny of blastomere C1 will show quenching effects before progeny of blastomere C4. To avoid quenching by hemoglobin, future studies on erythropoiesis should employ blue fluorescent proteins. Finally, our experiments are subject to sampling error, as demonstrated in Table 3. The embryo injected in blastomere C1 that showed 0% GFP-positive blood when sampled by tail bleed had circulating, GFP-positive cells. For these reasons, our attempts to quantify contributions to blood (Table 3) are usually underestimates. We emphasize that in mapping by frequency distribution, we can make no conclusions concerning mass contributions of specific blastomeres to the circulating blood at stage 41. The results in Table 4 suggest that blood circulating by stage 38 is derived from the dorsal half of the embryo, so contributions from column 1 and 2 blastomeres should not be considered trivial. From the data in Tables 2, 3 and 4, we conclude only that primitive blood descends from a widespread area of the cleavage stage embryo,

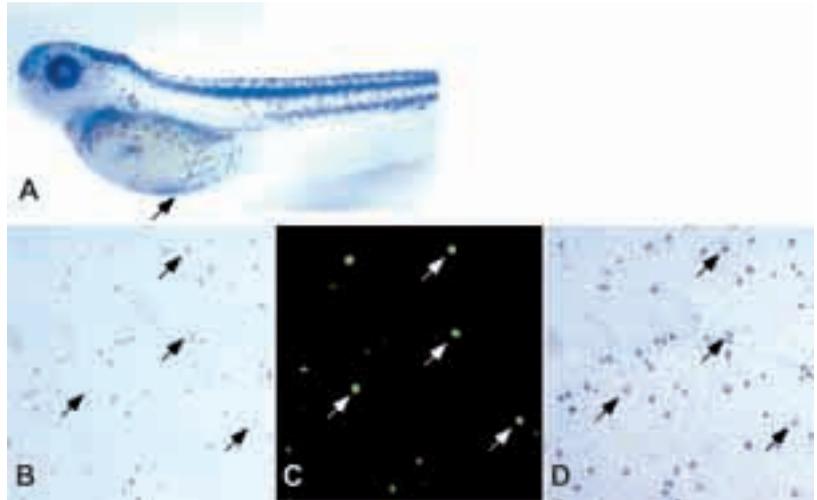


Fig. 4. Circulating, fluorescent cells are erythrocytes. (A) A control embryo, stage 40, stained for hemoglobin with benzidine. Vessels such as the vitelline veins (arrow) are heavily labeled. (B) Circulating cells collected by tail bleed from a C1-injected embryo. (C) A subset of collected cells express GFP. All GFP-positive cells stain with benzidine (D), indicating they contain hemoglobin.

and not strictly from the region currently called the ventral marginal zone.

A-tier contributions to blood

A4 and A3 progeny form the epidermal covering of the ventral blood islands, and some of their progeny enter the circulation at low, but reproducible, frequencies. Based on experiments with labeled recombinates of animal cap and marginal zone, Maeno et al. (1992) concluded that animal cap cells (which include A4 and A3 progeny), induce, but do not themselves form blood. We determined that A4-derived, circulating cells are erythrocytes, and not cells inadvertently swept into circulation with blood cells, by demonstrating that GFP-positive cells stain for hemoglobin. We conclude that progeny of animal tier cells *in vivo* can receive sufficient signals to differentiate as blood.

Comparison with previous fate maps

The map in Fig. 6A differs significantly from the summary diagrams currently in use (e.g., Fig. 1A,B; Heasman, 1997; Turpen et al., 1997). Two problems with previous diagrams deserve attention. First, the origins of primitive blood were not determined, and its assignment solely to the ventral marginal zone is unwarranted. Second, the imposition of summary mapping data onto the boundary lines of the original Keller fate map grossly misrepresents quantitative data describing the origins of somitic mesoderm. From Table 3 in Dale and Slack (1987a), the combined contribution from blastomeres B2 and C2 is 33% of the total volume of the somites at stage 30; B3 and C3 contribute 29%; B4 and C4 contribute 28%. Thus, B4 and C4 contribute nearly as much somitic tissue at stage 30 as B2 and C2. This warrants illustration of the somite territory in the marginal zone as a band of similar width extending to the ventral midline, as in Keller's revised map (Fig. 1C).

The value of constructing multiple fate maps for vertebrate embryos merits explanation. No single fate map is sufficient to describe such complex organisms, and multiple fate maps for all species used as model systems should be generated. Numerous maps are available for *Xenopus*, and differences between extant maps exist. These differences rarely represent mistakes by investigators, but result simply because investigators map to different stages. These observed

differences are useful in analyzing development. Vertebrate embryos form in an anterior-to-posterior sequence, so comparison of maps constructed from one stage (e.g., stage 6) to successively later stages (stage 22, Nakamura and Kishiyama, 1971; stage 30, Dale and Slack, 1987a; stage 32-34, Moody, 1987b) reveals the origins of tissues added at the posterior end of the forming embryo.

If we compare these maps for notochordal, somitic or neural territories, we see a common trend. Each territory expands progressively at successively older stages. As an example, we consider the somites. At stage 22, only the anterior 9-10 pairs of somites have formed (Nieuwkoop and Faber, 1967), and they arise from blastomeres C2 and C3 (Nakamura and Kishiyama, 1971). At stage 30, the anterior 25 pairs of somites are present, and they arise mainly from blastomeres C2, C3 and C4, with smaller contributions from blastomeres B2, B3 and B4 (Dale and Slack, 1987a). At stage 34, there are about 32 pairs of somites, and these arise from all A- and D-tier blastomeres in addition to B- and C-tier blastomeres (Moody,

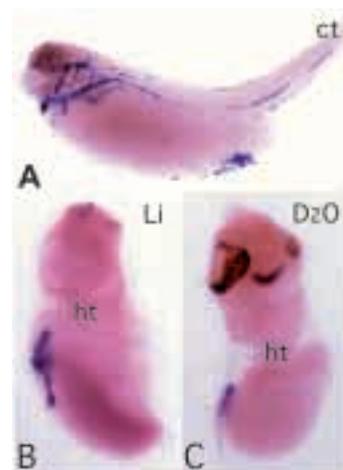


Fig. 5. In situ hybridization for α T3 globin at stage 40. (A) Control embryo. Cells within the vasculature and the posterior VBI express globin. The anterior VBI has already disintegrated. (B) Li^+ -generated, DAI 9-10 embryo expresses globin. (C) D_2O -generated, DAI 10 embryo, with its radial heart (ht), expresses globin.

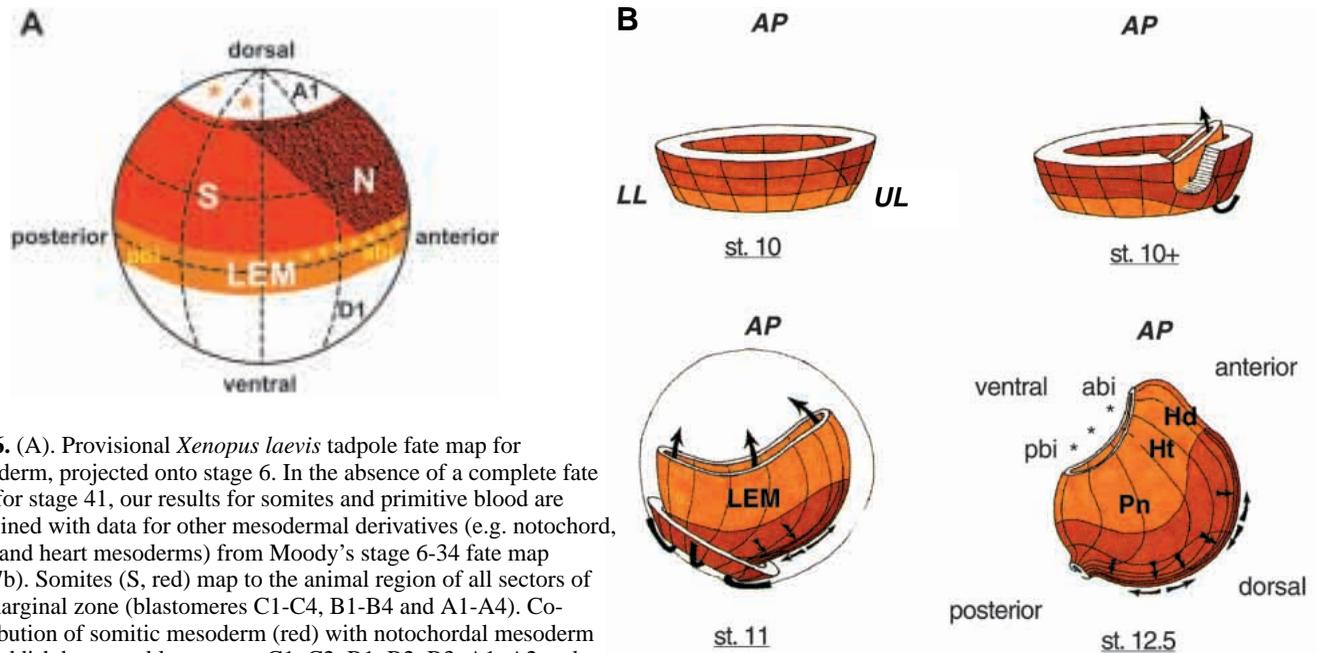


Fig. 6. (A). Provisional *Xenopus laevis* tadpole fate map for mesoderm, projected onto stage 6. In the absence of a complete fate map for stage 41, our results for somites and primitive blood are combined with data for other mesodermal derivatives (e.g. notochord, head and heart mesoderms) from Moody's stage 6-34 fate map (1987b). Somites (S, red) map to the animal region of all sectors of the marginal zone (blastomeres C1-C4, B1-B4 and A1-A4). Co-distribution of somitic mesoderm (red) with notochordal mesoderm (N, reddish brown – blastomeres C1, C2, B1, B2, B3, A1, A2 and A3) is indicated by the mixed coloring of the notochordal territory.

Blood arises from leading edge mesoderm (orange), situated in the vegetal region of the marginal zone, with a variable contribution from blastomeres A4 and A3, indicated by orange asterisks. Anterior leading edge mesoderm, including head and heart mesoderms (yellow asterisks) and the anterior portion of the ventral blood islands (abi), arises from C1, C2, D1 and D2 blastomeres. Posterior leading edge mesoderm, including the posterior region of the ventral blood islands (pbi) and probably the pronephros, arises from blastomeres C3, C4, D3 and D4. As there is no clear evidence at present that leading edge mesoderm cells are specified as particular cell types (e.g., blood) while residing in the vegetal region of the marginal zone of pre-gastrula embryos, the abbreviations for tissues shown within the LEM territory are meant to indicate general, not exact, distributions of the prospective tissues. For example, the precise relationship between the prospective head and heart mesoderms is unknown. They lie near the upper blastopore lip, but whether both mesoderms reach the median, or whether head mesoderm lies at the median while heart mesoderm resides more laterally, is not known. (B) Morphogenesis of the leading edge mesoderm (after Keller, 1991), including the prospective blood islands. Only the marginal zone is shown, from stage 10 to ca. stage 12.5. The animal pole (AP) is held in a fixed position. Dorsal mesoderm is red and leading edge mesoderm (comprised of head, intermediate and lateral plate mesoderms) is orange. Migration of the leading edge mesoderm and involution of the dorsal mesoderm begin at the upper lip around stage 10, and spreads to the lower lip by stage 10.5. Asterisks mark the future position of the ventral blood islands at the ventral midline, where the vanguard of the migrating leading edge coalesces into the blood islands. See text for fuller explanation. abi, anterior ventral blood islands; AP, animal pole; UL, upper lip; LL, lower lip; Hd, prospective head mesoderm; Ht, prospective heart mesoderm; pbi, posterior ventral blood islands; Pn, prospective pronephros.

1987b). At successively later stages, the territory expands as investigators literally map more somites. Comparison of the maps gives us a rough guide for the formation of somites: the anterior 9-10 somites form from blastomeres C2 and C3 by stage 22; trunk somites 10-25 arise mainly from C4, B2, B3 and B4 during the interval between stage 22 and stage 30 (determined by subtracting the blastomeres identified by Nakamura and Kishiyama from the blastomeres identified by Dale and Slack). Somites 25-32, which form between stage 30 and stage 34, arise mainly from B- and A-tier progeny (determined by subtracting the territory identified by Dale and Slack from the territory identified by Moody). This approach is 'rougher' for muscle than notochordal or neural tissues because somite morphogenesis is especially complex, with dorsal and ventral aspects of individual trunk/tail somites having separate origins from the central domain next to the notochord (Cooke and Webber, 1985; Moody, 1987b). Despite this complexity, the approach works. If we compare the information generated by this approach with the morphogenesis observations of Shih and Keller (1992) on shaved marginal zones, the two methods concur as to the

pattern in which the anterior-posterior aspect of the dorsal, axial structures is constructed. Thus, the existence of multiple maps provides an independent means to confirm observations made using other techniques. The information generated by multiple maps reveals the overwhelming complexity of the amphibian embryo, but the maps also give us the means to reduce the complexity into usable information. Map construction in *Xenopus* should continue, especially as interest shifts to organogenesis, and many organ primordia are not currently mapped.

Morphogenesis of the ventral blood islands

We find that C-, D- and sometimes A-tier blastomeres contribute to primitive blood. C- and D-tier cells contribute in a spatial and temporal succession, beginning with column 1 blastomeres (i.e., C1 and D1) at stage 35, and progressing through column 2 and 3 blastomeres to column 4 by stage 41.

In *Xenopus*, cells destined to form head, intermediate (e.g., pronephros), and lateral plate mesoderm (e.g., blood and heart) migrate as leading edge mesoderm (Keller, 1991). Leading edge mesoderm forms from the deep, vegetal region of the

marginal zone, immediately beneath the prospective bottle cells in the epithelial layer, and are derived from C- and D-tier blastomeres at stage 6. In Keller's morphogenesis diagrams (1991, see Figs 1C and 6B), leading edge mesoderm is depicted in orange, as opposed to the prospective notochord and somites, which are shown in red.

The migratory path of a leading edge mesoderm cell varies with its point of origin in the marginal zone, but all ultimately migrate towards the progeny of the A4 and A3 blastomeres in the animal cap. CD1-derived leading edge mesoderm migrate directly anteriorward, past the anterior neural plate, anterior neural folds, and prospective cement gland (which are primarily A1 progeny), to arrive in the regions of, and form, the heart and liver diverticulum situated along the ventral midline (Fig. 6B). The vanguard of this wave of migrating cells forms the anterior region of the ventral blood islands (asterisks). CD2-, CD3- and finally CD4-derived leading edge mesoderm cells do not migrate directly towards the blastocoel roof from their starting points in the vegetal end of the marginal zone. Their migratory paths are deflected somewhat dorsally by the morphogenetic activities of the prospective notochord and somites (Keller, 1976; see Fig. 6B). The leading edge cells then migrate ventrally from their relatively dorsal positions, and the vanguard of these cells arrives near the ventral midline, at positions posterior to the CD1 progeny and deep to the overlying A4/A3-derived epidermis (asterisks, Fig. 6B). As they arrive at the ventral midline, they coalesce into the mid-axial (CD2- and CD3 progeny) and posterior (CD4 progeny) regions of the blood islands. The progressive addition of cells to the blood islands is reflected in the progressive appearance of circulating blood cells in the tadpole. CD1-derived blood cells appear by stage 36 with CD2-, CD3- and CD4-derived blood appearing sequentially later. Thus, there is an A-P progression in blood production by the VBI that correlates with the cleavage-stage origins of the VBI around the circumference of the marginal zone (Fig. 6B): CD1 progeny form the anterior end of the ventral blood islands and CD4 progeny form the posterior end of the blood islands.

Although blood maps to the dorsal marginal zone in vivo, blood is not observed in dorsal marginal zone explants (Maeno et al., 1992; Kelley et al., 1994). A consideration of blood island morphogenesis and the signaling events involved in hematopoiesis may explain this observation. Blood cell development requires BMP-like signals (Zhang and Evans, 1996), and the BMP-4 antagonist noggin represses at least one factor required for blood differentiation; GATA-2 (Walmsley et al., 1994). From these results, one would predict that cells surrounded by BMP antagonists would be unable to differentiate as blood. Migration away from the prospective dorsal mesoderm and towards animal cap progeny in the embryo moves leading edge mesoderm cells out of a region believed rich in BMP antagonists (e.g., chordin, noggin), to an area of high BMP activity. As leading edge cells migrate towards the blastocoel roof, they express GATA-2, a marker of several lineages including hematopoietic cells (Bertwistle et al., 1996), and the gene repressed by noggin. Within explanted dorsal marginal zones, leading edge mesoderm cells may be unable to escape from BMP antagonists to an area of high BMP activity and differentiate as blood. Alternatively, cells within the marginal zone may need to contact animal cap cells for inductive signals other than BMP (Maeno et al., 1994), and this

interaction cannot occur in explanted dorsal marginal zones that lack animal cap tissue. Both possibilities are supported by the results of Tracey et al. (1998), who demonstrated that a marker of blood tissue (*Xaml-1*) is expressed in explanted dorsal marginal zones that retain animal cap tissue.

Reorienting the dorsal-ventral axis in *Xenopus*

The new map forces reconsideration of patterning within the marginal zone. Examining the map, we ask, 'From which regions of the stage 6 embryo does dorsal mesoderm arise, and from which regions does ventral mesoderm arise?' We define 'dorsal mesoderm' as notochordal and somitic mesoderm, which lies in immediate proximity to the dorsal midline of the tadpole, and 'ventral mesoderm' as all other mesoderms which lies away from the dorsal midline in the tadpole, including intermediate, lateral plate and head mesoderms. We see that dorsal mesoderms occupy the animal region of the marginal zone through all 360°, while ventral mesoderms map to the vegetal portion of the marginal zone. Likewise, dorsal endoderm (i.e., the archenteron roof) arises from the epithelium covering the animal region of the marginal zone, and ventral endoderm (i.e., the archenteron floor) arises from cells located vegetal of the bottle cells (Morgan, 1897; Keller, 1975). Thus, dorsal or ventral for both mesoderm and endoderm is established across the animal/vegetal aspect of the marginal zone (Fig. 6A), not around the circumference. This represents a 90° rotation of the dorsal-ventral axis from current interpretation of tailbud fate maps.

Implications of the tadpole map for the three-signal hypothesis of amphibian mesoderm formation

In the standing model of mesoderm induction, the three-signal hypothesis (Smith and Slack, 1983; Dale and Slack, 1987b), two signals from the vegetal hemisphere of the blastula induce the overlying animal hemisphere to form mesoderm. Dorsovegetal cells (the Nieuwkoop center) induce overlying cells to form Spemann's organizer (signal 1), which in turn forms dorsal mesoderm. Ventro-vegetal cells induce mesoderm of ventral character (blood, mesenchyme and mesothelium, reviewed in Smith, 1989) in the ventral and lateral marginal zones (signal 2). During gastrula stages, a third signal from Spemann's organizer dorsalizes ventral mesoderm in the lateral marginal zone to form somitic mesoderm.

The revised fate map challenges this model. One critical problem is with signal 1 from the Nieuwkoop center. Dorsovegetal cells supposedly induce dorsal mesoderm (i.e., notochord) in overlying tissue, but prospective blood (as well as heart and head mesoderms) lie between the dorsovegetal signaling cells and the notochordal territory. The model cannot explain how extreme ventral and dorsal mesoderms arise from the same meridians, especially the dorsal midline, where cells inherit putative 'dorsal determinants' as a result of cortical rotation. Formation of blood and notochord by C1 progeny forces us to question whether cortical rotation, operating via the Nieuwkoop center, leads to dorsal mesoderm formation. We return to this question below.

The second signal in the three-signal model proposes ventro-vegetal cells of the blastula induce ventral mesoderm in overlying cells, and offers no explanation for blood formation by C1-, A4- or A3-progeny. These cells are widely separated geographically in blastulae, when the model proposes the

putative inducing signal is passed. A simpler hypothesis is that the signal(s) for blood differentiation comes later, when these initially widespread tissues coalesce into the blood islands near the ventral midline.

Recent observations on gene expression patterns in the marginal zone are more complex than the three-signal model predicts. Namely, Spemann's organizer expresses mRNAs encoding separate dorsalizing and ventralizing activities [e.g., dorsalizing activities include *noggin* (Smith and Harland, 1992) and *chordin* (Sasai et al., 1994), and ventralizing activity anti-dorsalizing morphogenetic protein (Moos et al., 1995)]. Likewise, the ventral marginal zone expresses mRNAs encoding separate ventralizing and dorsalizing activities (e.g., *Xwnt8*, Christian et al., 1991; Smith and Harland, 1991; *Xvent1*, Gawantka et al., 1995; *szl*, Salic et al., 1997). A 'ventral organizer' in the ventral marginal zone is proposed to antagonize Spemann's organizer (Salic et al., 1997), with interplay between the two determining dorsal-ventral patterning around the circumference of the marginal zone. As our map indicates dorsal-ventral patterning is determined across the animal-vegetal aspect of the marginal zone, we suggest an alternative interpretation. We propose that the region of Spemann's organizer contributes to mesoderms as diverse as notochord and blood because both dorsalizing and ventralizing factors are present concomitantly. Similarly, throughout the marginal zone, dorsal (muscle) and ventral (blood) mesoderm are able to form because both dorsalizing and ventralizing factors are present at all meridians.

Redefining the regions of the marginal zone

It is customary to determine the orientation of the primary embryonic axes in early embryos by fate mapping. This was not the case in amphibians. The phrase 'dorsal lip of the blastopore' was in use in the literature (Morgan, 1897) many years before a fate map was constructed for an amphibian embryo (Vogt, 1929). The designation of Spemann's 'upper lip' as the 'dorsal lip', and his 'lower lip' as the 'ventral lip', led to phrases such as dorsal marginal zone and ventral marginal zone to refer to different meridians on the equator of the embryo. The revised map reveals that these phrases do not reflect accurately the fates of cells in the regions of the embryo to which they are applied. Formation of heart and head mesoderm (both situated in the *anterior*, ventral region of the tadpole), the *anterior* end of the ventral blood islands and the *anterior* notochord by C1 progeny suggests that the Nieuwkoop center acts in a pathway to generate *anterior* mesoderm, of both *dorsal* and *ventral* qualities. In conjunction with our earlier observation that the region currently called the ventral marginal zone would more appropriately be called the posterior marginal zone, we suggest that the region including Spemann's organization center, which is currently called the 'dorsal marginal zone', would be named more accurately the 'anterior marginal zone', since anterior mesodermal and endodermal tissues arise from this region. Spemann's upper lip of the blastopore is the 'anterior lip', and his lower lip is the 'posterior lip'. If we believe that, by definition, the dorsal marginal zone should give rise to dorsal mesoderm, and the ventral marginal zone should give rise to ventral mesoderm, then the new map reveals that the animal portion of the marginal zone is the dorsal marginal zone, and the vegetal portion is the ventral marginal zone.

Although nomenclature changes will cause confusion in the immediate future, these designations more accurately describe the embryo at all stages, as they unify the meaning of 'dorsal' and 'ventral' in pre- and post-gastrula stage *Xenopus* embryos. Ultimately the proposed nomenclature changes will clarify our view of the amphibian embryo and facilitate studies to unravel the mechanisms of vertebrate axial patterning.

Conservation within the phylum Chordata

Finally, our revision of the fate and axis orientation maps establish congruence between these maps for a vertebrate species and those for the urochordates and cephalochordates. Close conservation of these maps between species in all sub-phyla of the Chordata suggests that mechanisms of axial patterning may be more highly conserved than is currently believed. Urochordates specify the dorsal-ventral axis during oogenesis and the anterior-posterior axis via a mechanism involving the sperm entry point during the first cell cycle (reviewed by Goldstein and Freeman, 1997). Based on inaccurate fate maps, it was believed that amphibians were different, specifying the anterior-posterior axis during oogenesis and the dorsal-ventral axis by cortical rotation (which is related to the sperm entry point). Our revisions connect cortical rotation with establishment of the anterior-posterior axis, revealing similarities between *Xenopus* and other chordates.

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REFERENCES

- Bertwistle, D., Walmsley, M. E., Read, E. M., Pizzey, J. A. and Patient, R. K. (1996). GATA factors and the origins of adult and embryonic blood in *Xenopus*: responses to retinoic acid. *Mech. Dev.* **57**, 199-214.
- Christian, J. L., McMahon, J. A., McMahon, A. P. and Moon, R. T. (1991). *Xwnt-8*, a *Xenopus Wnt-1/int-1*-related gene responsive to mesoderm inducing factors, may play a role in ventral mesodermal patterning during embryogenesis. *Dev.* **111**, 1045-1056.
- Cooke, J. and Smith, E. (1988). The restrictive effect of early exposure to lithium upon body pattern in *Xenopus* development, studied by quantitative anatomy and immunofluorescence. *Dev.* **102**, 85-99.
- Cooke, J. and Webber, J. A. (1985). Dynamics of the control of body pattern in the development of *Xenopus laevis*. I. Timing and pattern in the development of dorsoanterior and posterior blastomere pairs, isolated at the 4-cell stage. *J. Embryol. Exp. Morph.* **88**, 85-112.
- Dale, L. and Slack, J. M. W. (1987a). Fate map for the 32-cell stage of *Xenopus laevis*. *Development* **99**, 527-551.

- Dale, L. and Slack, J. M. W.** (1987b). Regional specification within the mesoderm of early embryos of *Xenopus laevis*. *Development* **100**, 279-295.
- Gawantka, V., Delius, H., Hirschfeld, K., Blumenstock, C. and Niehrs, C.** (1995). Antagonizing the Spemann organizer: role of the homeobox gene *Xvent-1*. *EMBO J.* **14**, 6268-6279.
- Goldstein, B. and Freeman, G.** (1997). Axis specification in animal development. *BioEssays* **19**, 105-116.
- Harland, R. M.** (1991). In situ hybridization: an improved whole mount method for *Xenopus* embryos. In *Methods in Cell Biology*, Vol. 36: (ed. B. Kay and H. B. Peng), pp. 685-695. Academic Press, San Diego.
- Heasman, J.** (1997). Patterning the *Xenopus* blastula. *Development* **124**, 4179-4191.
- Heim, R. and Tsien, R.** (1995). Engineering green fluorescent protein for improved brightness, longer wavelengths and fluorescence resonance energy transfer. *Curr. Biology* **6**, 178-182.
- Hemmati-Brivanlou, A. and Thomsen, G. H.** (1995). Ventral mesodermal patterning in *Xenopus* embryos: expression patterns and activities of BMP-2 and BMP-4. *Dev. Genet.* **17**, 78-89.
- Kao, K. and Elinson, R. P.** (1988). The entire mesodermal mantle behaves as Spemann's organizer in dorsoanterior enhanced *Xenopus laevis* embryos. *Dev. Biol.* **127**, 64-77.
- Kau, C.-L. and Turpen, J. B.** (1983). Dual contribution of embryonic ventral blood island and dorsal lateral plate mesoderm during ontogeny of hematopoietic cells in *Xenopus laevis*. *J. Immunol.* **131**, 2262-2266.
- Keller, R. E.** (1975). Vital dye mapping of the gastrula and neurula of *Xenopus laevis*. I. Prospective areas and morphogenetic movement of the superficial layer. *Dev. Biol.* **42**, 222-241.
- Keller, R. E.** (1976). Vital dye mapping of the gastrula and neurula of *Xenopus laevis*. II. Prospective areas and morphogenetic movements of the deep layer. *Dev. Biol.* **51**, 118-137.
- Keller, R.** (1991). Early embryonic development of *Xenopus laevis*. In *Methods in Cell Biology*, Vol. 36: *Xenopus laevis*: Practical uses in cell and molecular biology (ed. B. Kay and H. B. Peng) Chapter 5, pp. 61-113. New York, Academic Press.
- Keller, R., Cooper, M. S., Danilchik, M., Tibbetts and Wilson, P.** (1989). Cell intercalation during notochord development in *Xenopus laevis*. *J. Exp. Zool.* **251**, 134-154.
- Kelley, C., Yee, K., Harland, R. and Zon, L. I.** (1994). Ventral expression of GATA-1 and GATA-2 in the *Xenopus* embryo defines induction of hematopoietic mesoderm. *Dev. Biol.* **165**, 193-205.
- Lemberg, R. and Legge, J. W.** (1949). *Hematin Compounds and Bile Pigments*. New York: Interscience Publishers.
- Maeno, M., Ong, R. C. and Kung, H.-F.** (1992). Positive and negative regulation of the differentiation of ventral mesoderm for erythrocytes in *Xenopus*. *Dev. Growth Differ.* **34**, 567-577.
- Maeno, M., Ong, R. C., Xue, Y., Nishimatsu, S.-I., Ueno, N. and Kung, H.-F.** (1994). Regulation of primary erythropoiesis in the ventral mesoderm of *Xenopus* gastrula embryo: evidence for the expression of a stimulatory factor(s) in animal pole tissue. *Dev. Biol.* **161**, 522-529.
- Moody, S. A.** (1987a). Fates of the blastomeres of the 16-cell stage *Xenopus* embryo. *Dev. Biol.* **119**, 560-578.
- Moody, S. A.** (1987b). Fates of the blastomeres of the 32-cell-stage *Xenopus* embryo. *Dev. Biol.* **122**, 300-319.
- Moos, M. Jr., Wang, S. and Krinks, M.** (1995). Anti-dorsalizing morphogenetic protein is a novel TGF- β homolog expressed in the Spemann organizer. *Development* **121**, 4293-4301.
- Morgan, T. H.** (1897). *The development of the frog's egg*. New York: MacMillan.
- Nakamura, O. and Kishiyama, K.** (1971). Prospective fates of the blastomeres at the 32 cell stage of *Xenopus laevis* embryos. *Proc. Japan Acad.* **47**, 407-412.
- Nieuwkoop, P. D.** (1969). The formation of mesoderm in Urodelean amphibians. I. Induction by the endoderm. *Roux's Archiv.* **162**, 341-373.
- Nieuwkoop, P. D. and Faber, J.** (1967). *Normal table of Xenopus laevis (Daudin)*. Amsterdam: North Holland.
- Salic, A., Kroll, K., Evans, L. M. and Kirschner, M. W.** (1997). *Sizzled*: a secreted Xwnt8 antagonist expressed in the ventral marginal zone of *Xenopus* embryos. *Development* **124**, 4739-4748.
- Sasai, Y., Lu, B., Steinbesser, H., Geissert, D., Gont, L. and De Robertis, E. M.** (1994). *Xenopus chordin*: A novel dorsalizing factor activated by organizer-specific homeobox genes. *Cell* **79**, 779-790.
- Scharf, S. R., Rowning, B., Wu, M. and Gerhart, J. C.** (1989). Hyperdorsoanterior embryos from *Xenopus* eggs treated with D₂O. *Dev. Biol.* **134**, 175-188.
- Shih, J. and Keller, R.** (1992). Patterns of cell motility in the organizer and dorsal marginal zone of *Xenopus laevis*. *Development* **116**, 915-930.
- Smith, J. C.** (1989). Mesoderm induction and mesoderm-inducing factors in early amphibian development. *Development* **105**, 665-677.
- Smith, J. C. and Slack, J. M. W.** (1983). Dorsalization and neural induction: properties of the organizer in *Xenopus laevis*. *J. Embryol. Exp. Morphol.* **78**, 299-317.
- Smith, W. C. and Harland, R. M.** (1991). Injected *Xwnt-8* RNA acts early in *Xenopus* embryos to promote formation of a vegetal dorsalizing center. *Cell* **67**, 753-765.
- 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**, 828-840.
- Spemann, H.** (1938). *Embryonic Development and Induction*. New Haven: Yale University Press.
- Tracey, W. D. Jr., Pepling, M. E., Thomsen, G. H. and Gergen, J. P.** (1998). A *Xenopus* homologue of *aml-1* reveals unexpected patterning mechanisms leading to the formation of embryonic blood. *Development* **125**, 1371-1380.
- Turpen, J. B., Kelley, C. M., Mead, P. E. and Zon, L. I.** (1997). Bipotential primitive-definitive hematopoietic progenitors in the vertebrate embryo. *Immunity*, **7**, 325-334.
- Vodicka, M. A. and Gerhart, J. C.** (1995). Blastomere derivation and domains of gene expression in the Spemann organizer of *Xenopus laevis*. *Development* **121**, 3505-3518.
- Vogt, W.** (1929). Gestaltungsanalyse am Amphibienkeim mit ortlicher Vitalfarbung. II. Teil Gastrulation und Mesodermbildung bei Urodelen und Anuren. *Wilhelm Roux Arch. Entwicklungsmech. Org.* **120**, 384-706.
- Walmsley, M. E., Guille, M. J., Bertwistle, D., Smith, J. C., Pizzey, J. A. and Patient, R. K.** (1994). Negative control of *Xenopus* GATA-2 by activin and *noggin* with eventual expression in precursors of the ventral blood islands. *Development* **120**, 2519-2529.
- Yamaguchi, Y. and Shinagawa, A.** (1989). Marked alteration at midblastula transition in the effect of lithium on formation of the larval body pattern of *Xenopus laevis*. *Dev. Growth Differ.* **31**, 531-541.
- Zernicka-Goetz, M., Pines, J., Ryan, K., Siemering, K. R., Haseloff, M. J., Evans, M. J. and Gurdon, J. B.** (1996). An indelible lineage marker for *Xenopus* using a mutated green fluorescent protein. *Development* **122**, 3719-3724.
- Zhang, C. and Evans, T.** (1996). BMP-like signals are required after the mid-blastula transition for blood cell development. *Dev. Genet.* **18**, 267-278.