Gata2 provides an early anterior bias and uncovers a global positioning system for polarity in the amniote embryo

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SUMMARY
The first axis to be specified during vertebrate development is that between the site where gastrulation will begin and the opposite pole of the embryo (dorsobranchial axis in amphibians and fish, anteroposterior in amniotes). This relies on Nodal activity, but different vertebrates differ in how this activity is positioned. In chick, the earliest known asymmetry is posterior expression of the TGFβ-related factor Vg1, close to the future Nodal expression domain. Here we show that the transcription factor Gata2 is expressed anteriorly before this stage. Gata2 influences the site of primitive streak formation and its role is independent from, and upstream of, Vg1 and Wnt. However, although Vg1 is required for streak formation, Gata2 does not act as an absolute anterior specifier, but provides an anterior bias. These findings point to previously unsuspected global determinants of polarity of the early amniote embryo.

KEY WORDS: Embryonic polarity, GATA factors, Chick embryo, Gastrulation, Primitive streak formation, Nodal, Vg1, Gdf-1, Embryonic regulation, Regeneration

INTRODUCTION
In most vertebrates, the site at which gastrulation is initiated is specified by cell interactions mainly involving Nodal-related signals in cooperation with the canonical (β-catenin-dependent) Wnt pathway. However, different animals differ in how they achieve the localisation of these signals at the correct location: in Xenopus embryos this is dependent on maternal determinants: nuclear localisation of β-catenin specifies ‘dorsal’ (Houston and Wylie, 2004), whereas maternal RNAs encoding the transcription factor VegT (Zhang and King, 1996; Zhang et al., 1998; Kofron et al., 1999; Mir et al., 2007) and the TGFβ-related factor Vg1 (Weeks and Melton, 1987; Thomsen and Melton, 1993; Birsoy et al., 2006) specify ‘vegetal’ identity. Their overlap defines the Nieuwkoop Centre, the first signalling centre of the embryo, which in turn induces the formation of the Spemann organizer at the site where gastrulation begins, the future dorsal lip of the blastopore. Induction of the Spemann organizer requires Nodal activity together with canonical Wnt. Similar interactions occur in teleosts, where Nodal- and Wnt-related signals specify the embryonic shield as the site where gastrulation begins at the margin of the embryo during early epiboly (Gritsman et al., 1999; Schier, 2003).

In amniotes, maternal determinants appear to exist, such as the δ-ooplas of the sub-blastodermal yolk (nucleus of Pander), which may determine asymmetries in formation of the primitive endoderm (Callebaut et al., 2000). However, these maternal factors must be much less important than in frogs and fish, because amniote embryos retain the ability to regulate (or ‘regenerate’ the entire body) for a very long time after fertilisation (Stern and Downs, 2012). For example, if a chick embryo is cut into several fragments right up to the start of primitive streak formation (when it may have 20,000-50,000 cells), each fragment can initiate the formation of a complete axis (Lutz, 1949; Spratt and Haas, 1960). This property can also account for the formation of monozygotic twins (including most types of conjoined, or ‘Siamese’, twins) in humans and other primates, which are thought to be able to arise late in development (Enders, 2002a; Kaufman, 2004), and for the obligate monozygotic quadruplets seen in some species of armadillo (Enders, 2002b; Eakin and Behringer, 2004). Apart from these cases, most amniote embryos generate only a single axis, implying that there must be mechanisms both to determine the orientation of the future axis and to prevent twinning. We still know relatively little about these mechanisms. It is generally believed that the posterior end of the embryo (from where the primitive streak will arise) contains the main signals.

The earliest known marker of embryonic polarity in the chick is transcriptional activation of Vg1, which is localised in the posterior marginal zone, the functional equivalent of the Nieuwkoop Centre (Azar and Eyal-Giladi, 1979; Kanher et al., 1985; Kanher and Eyal-Giladi, 1986; Kanher and Eyal-Giladi, 1989; Shah et al., 1997; Bachvarova et al., 1998; Kanher, 1998; Bertocchini and Stern, 2002; Bertocchini et al., 2004; Stern, 2004). In turn, Vg1 and Wnt cooperate to induce transcription of Nodal around the site where the primitive streak arises (Skromne and Stern, 2001; Skromne and Stern, 2002; Stern, 2004). In the mouse Nodal transcripts are not localised posteriorly, but the position of primitive streak formation relies on the removal of Nodal antagonists (Cerberus-like and Lefty1) from the site at which the streak will arise, due to migration of the visceral endoderm (Perea-Gomez et al., 2001; Perea-Gomez et al., 2002; Tam and Gad, 2004; Yamamoto et al., 2004; Stern and Downs, 2012). Thus, localisation of Nodal activity at the site where gastrulation is initiated is the common feature of all vertebrates. Partly for this reason it is generally assumed that the main initial determinants of polarity must reside close to this region (‘dorsally’ in amniotes, ‘posteriorly’ in anamniotes).

Here we show that the transcription factor Gata2 is expressed at the anterior (‘anti-gastrular’) pole in the chick before Vg1 appears posteriorly. Gata2 influences the site of primitive streak formation and its role is independent from, and upstream of, Vg1 and Wnt. Gata2 does not act as an absolute anterior specifier, but rather provides an anterior bias for embryo polarity and appears to
cooperate with other factors to position Vg1 expression posteriorly. These findings point to previously unsuspected global determinants of polarity of the early amniote embryo and suggest that the earliest asymmetry in the chick embryo may reside anteriorly rather than close to the site of primitive streak formation.

MATERIALS AND METHODS

Eggs and embryos
Fertile hens’ eggs were obtained from Henry Stewart (UK) and Granja Gibert (Spain) (Brown Bovan Gold) and staged in Roman numerals for pre-primitive streak stages (Eyal-Giladi and Kochav, 1976) and in Arabic numerals (Hamburger and Hamilton, 1951) starting from stage 2, when the primitive streak appears. Embryos were cultured in modified New culture (New, 1955; Stern and Ireland, 1981).

Gene expression studies
In situ hybridisation and whole mount immunohistochemistry were carried out as described (Stern, 1998) using probes: chick Bmp4 (Liem et al., 1995), brachyury (Kispet et al., 1995a; Kispet et al., 1995b; Knezevic et al., 1997), Gata2 and Gata3 (Sheng and Stern, 1999), Nodal/cNR-I (Levin et al., 1995), Vg1 (Shah et al., 1997), Wnt8c (Hume and Dodd, 1993) and Chordin (Streit et al., 1998).

Gain- and loss-of-function experiments
Fluorescein-labelled morpholinos (MOs) against Gata2, Gata3, Vg1 and a standard control MO (Gene Tools) were delivered to young embryos by electroporation as described (Voiculescu et al., 2007; Voiculescu et al., 2008). Gata2-MO was designed to target the first splicing site: 5'-GGGATGCTCATT-TACCGTGTGCCTG-3' (Linker et al., 2009). Vg1-MO targeted the initial ATG: 5'-AGACCTCCATCTTCGCCGG-3' (Linker et al., 2009). Vg1-MO was designed to target the initial ATG: 5'-GAGGCCACCATGCACATGCC-3'. For Vg1 misexpression experiments, we transplanted COS cells transfected with a cVg1-dorsalin construct; pellets of 1000 cells were generated from hanging drops and grafted into host embryos as previously described (Shah et al., 1997; Streit et al., 1998; Skromne and Stern, 2001; Skromne and Stern, 2002).

RESULTS

Vg1 is required for axis formation
Previous studies (Seleiro et al., 1996; Shah et al., 1997) revealed that the gene encoding Vg1, a Nodal/Activin-related protein, is normally expressed in the posterior marginal zone of the chick embryo, and that its misexpression elsewhere in the marginal zone is sufficient to induce the formation of an ectopic, complete embryonic axis. However, it is unknown whether Vg1 is essential for normal axis formation. To assess this we electroporated Vg1 morpholino in the normal expression domain of this gene (Fig. 1A). While control MO-electroporated embryos were unaffected (n=21 embryos with abnormalities; Fig. 1B), Vg1 morpholino severely affected axis formation. In 9/23 (39%) embryos, the primitive streak arose from one or more ectopic sites, either adjacent to the original posterior (Fig. 1E,F), or from multiple sites, sometimes resulting in brachyury expression. In the absence of other clear features of polarity in addition to Vg1. The transcription factors Gata2 and Gata3 are good candidates because they are expressed anteriorly at primitive streak and earlier stages (Sheng and Stern, 1999). We therefore examined the timecourse of Gata2, Gata3 and Vg1 expression by in situ hybridisation. To obtain very young embryos we used eggs laid in winter, some of which are at stages VIII-IX (Eyal-Giladi and Kochav, 1976). At these stages, Gata2 is expressed weakly and ubiquitously in the area pellucida, marginal zone and inner part of the area opaca (Fig. 2A). After a few hours’ incubation (stage IX), expression becomes more asymmetric, clearing from one side of the posterior area pellucida, marginal zone and area opaca but remaining elsewhere (Fig. 2B). In the absence of other clear
markers of polarity we cannot be certain of the orientation of these embryos, but at stage X, Gata2 expression becomes more obviously graded, with its highest level anteriorly, decreasing posteriorly and with no expression visible in the posterior part of the embryo (Fig. 2C). By stage XIII, Gata2 transcripts appear in the posterior extra-embryonic region as well, although at low levels (not shown) (see Sheng and Stern, 1999). At early streak stages, the Gata2 expression domain includes the posterior primitive streak (Sheng and Stern, 1999). Vg1 expression is not detectable until stage X, when it is confined to the posterior marginal zone (Fig. 2D), where it remains until primitive streak formation; thereafter it is expressed in the posterior streak (Shah et al., 1997). Comparison between Gata2 and Vg1 at stages X-XIII therefore reveals striking complementarity, with Vg1 expressed in the posterior marginal zone, where Gata2 is absent but at later stages the two genes partly overlap in the posterior streak (not shown). Gata2 transcripts were reported to be expressed anteriorly but not until stage 4 (Sheng and Stern, 1999). To confirm this we examined embryos at earlier stages; we did not detect any expression before the primitive streak stage (supplementary material Fig. S1). These observations make Gata2 a good candidate determinant of anterior polarity.

Gata2 influences the polarity of axis formation

To test the hypothesis that Gata2 may act as an anterior determinant, we electroporated MO against Gata2 into the normal expression domain of this gene (Fig. 3A). Five out of 46 embryos (11%, Fig. 3C) displayed a double primitive streak and a further 15/46 (33%) contained a single streak arising from an ectopic site (in some cases the remnant of the original streak was still visible as a faint brachyury-expressing region; e.g. Fig. 3B). By contrast, most embryos electroporated with control morpholinos were normal (Fig. 3D) (90/92; one had a displaced streak and one had a double streak). It is conceivable that Gata3 may partly compensate for Gata2 in this experiment. To cover this possibility, we co-electroporated MOs directed against Gata2 and Gata3. This enhanced the effect compared with Gata2-MO alone, but only slightly [3/15 (20%) with double streaks, 5/15 (33%) with displaced streak]. Although these results suggest that Gata2/3 factors are anterior determinants of embryonic polarity, the position of the ectopic axes forming in this experiment suggest more complexity than the idea of Gata2/3 being a simple inhibitor. Indeed, in about half of the embryos with an ectopic streak, the streak arose not in the middle of the domain where Gata2 had been knocked down, but at one edge of this domain (e.g. Fig. 3B).

The formation of ectopic streaks in GATA knockdown experiments could be explained either by GATA factors acting upstream of other factors known to be involved in primitive streak formation, such as Vg1 and Wnt8C and their target Nodal (Hume and Dodd, 1993; Levin et al., 1995; Skromne and Stern, 2001; Bertocchini and Stern, 2002; Skromne and Stern, 2002). Alternatively, GATA may act downstream of these factors or through an unrelated mechanism. To test this, we examined the expression of Vg1, Wnt8C and Nodal 6-8 hours after electroporation of Gata2/3-MOs. Vg1 was expressed ectopically (Fig. 3E-G); occasionally, it was upregulated all around the marginal zone. Wnt8C was also expressed ectopically (Fig. 3H-J), losing its posterior bias and occasionally upregulated all around. Of these markers were affected 6 hours after Gata-MO electroporation: for Vg1, 21/52 (40%) embryos showed ectopic expression and 6/52 (11.5%) had no expression (1/54 controls had ectopic Vg1 expression, the rest were normal). For Wnt8C 5/13 (38%) had ectopic expression (Fig. 3G, 1/35 controls had ectopic expression; Fig. 3I, 5/35 (14%) had no expression). Nodal transcripts were also affected but only after 8 hours following Gata2/3-MO electroporation (7/17, 41% with ectopic expression, compared with 1/51 controls with ectopic expression and 2/51 with no expression) (Fig. 3K-M). Finally, it is possible that GATA knockdown could either induce an ectopic Koller’s sickle, or somehow attract sickle cells from the posterior end of the embryo. Although unlikely, we tested this using Chordin as a marker for the sickle after electroporation of Gata2/3-MO; neither ectopic expression nor extension of the endogenous domain was seen (supplementary material Fig. S2). Together, these results suggest that Gata2/3 factors act upstream of Vg1/Wnt and Nodal. GATA may therefore influence polarity in the early embryo, perhaps contributing to position the initial expression of Vg1 and Wnt8C in their normal domains.

Gata2/3 and embryonic regulation

When a pre-streak-stage embryo is cut into anterior and posterior halves and these are cultured separately, both can generate an embryonic axis (Lutz, 1949; Spratt and Haas, 1960; Bertocchini et al., 2004). The anterior half does not express Vg1 at the time of cutting but Vg1 begins to be transcribed after 8-9 hours at either the left or the right edge of the margin adjacent to the cut (Bertocchini et al., 2004). That this new expression domain is restricted to the most posterior part of the anterior half is consistent with the idea presented above that the graded expression of GATA factors (decreasing posteriorly at early stages) might act as an anterior determinant, regulating the expression of Vg1. The anterior half of an embryo should therefore constitute a sensitised assay for testing the role of GATA factors: we expected GATA knockdown at the most anterior edge of an isolated anterior half to cause the formation of an axis within the area where GATA has been knocked down. Surprisingly, electroporation of Gata2-MO at the anterior edge of an isolated anterior half does not cause a primitive streak to form from the anterior electroporated cells, but still appears from one side of the anterior half [16/29 (55%) with an axis from a lateral region, 13/29 (45%) with no streak] (Fig. 4A-C). Like the previous experiments, this result suggests that GATA provides a bias for polarity but is not an absolute determinant.

To test this bias in a different assay, we tested the effect of Gata2 knockdown on one side of the posterior edge of the anterior half. If our hypothesis is correct we would expect primitive streak formation from the electroporated site, where Gata is downregulated. Indeed, the primitive streak forms mainly from the electroporated side: 13/17 (76%) embryos had a streak arising from the electroporated side, 2/17 had no streak, 1/17 formed two streaks and 1/17 formed a streak from the opposite side (Fig. 4D-F).
contrast, anterior halves electroporated with a control MO showed no lateral bias: 6/34 (18%) formed a streak from the same side, 7/34 (21%) formed a streak from the opposite side, 17/34 (50%) had no streak and 3/34 (9%) developed two streaks. As an additional test of the role of Vg1 in axis formation in isolated halves, we electroporated Vg1 MO on one side of the anterior half – if Vg1 is important, this would be expected to produce a streak mainly from the opposite side. In the experiment, 9/20 (45%) produced a streak from the opposite side, 4/20 (20%) did not form a streak, 2/20 (10%) formed two streaks and 3/20 (15%) formed a streak from the electroporated side (not shown). Thus, Gata2 and Vg1 have opposite effects as determinants of embryo polarity, but the effects of Gata2 are stronger during embryonic regulation (regeneration) than in normal, intact embryos.

**Gata2 and Vg1 expression controlled by a ‘global positioning system’**

The above findings suggest that Gata2 is initially expressed as a gradient, strongest anteriorly, before the appearance of Vg1 transcripts in the posterior marginal zone. Then (stages XI-XII), Gata2 transcripts clear from the posterior domain where Vg1 is expressed. This raises the possibility that at this stage, Vg1 inhibits Gata2 expression locally. To test this we misexpressed Vg1 in the anterior marginal zone, and studied the effect on Gata2 expression after 4-8 hours of incubation: Gata2 expression was unaffected (0/27) (not shown), suggesting that Gata2 expression is independent from Vg1.

In an isolated anterior half, Vg1 expression appears on one side of the posterior edge about 9 hours after cutting (Bertocchini et al., 2004). Does Gata2 expression also change in the anterior half? Surprisingly, Gata2 expression becomes radial in the marginal zone/area opaca before Vg1 is activated (about 6-7 hours after cutting) (Fig. 4H); at 9 hours, Gata2 expression becomes undetectable on one side of the posterior region, where some cells of the embryonic area start to express Chordin, a marker for the organizer and its precursor cells (Fig. 4I). By 11 hours (when the axis starts to form), Chordin expression becomes localised in a region of the area pellucida facing the zone devoid of Gata2 expression (Fig. 4J). This result suggests that Gata2 is radially expressed at first, but downregulated at about the same time as the onset of Vg1 expression. Thus, the isolated anterior half appears to recapitulate the events of very early stages of development (Fig. 2, at stages VIII-XI). Although Vg1 and Gata2 appear to influence each other to some extent, the onset and maintenance of their expression seem to be controlled independently, suggesting the existence of a ‘global positioning system’ throughout the embryo, responsible for localising the domains of expression of both genes.

**GATA acts through secreted factors of the TGFβ family**

The non-cell-autonomous effects of Gata2-knockdown (e.g. Fig. 3B) suggest that expression of a secreted factor might be controlled by GATA. One possibility is that Vg1 expression is repressed by GATA. To test this, we analysed Vg1 expression after electroporating GATA-MO on one side of an isolated anterior half (Fig. 4D): 8/22 (36%) cases showed Vg1 expression only from the electroporated side (supplementary material Fig. S3A,B), none from the opposite side (0%), four had expression on both sides (18%), three showed diffuse, weak expression (14%) and seven showed no expression (32%). After control-MO electroporation 3/12 (25%) anterior halves showed Vg1 expression on the same side, three from the opposite side (25%), one on both sides (8%), and five (42%) showed no expression. These results suggest that Gata2/3 knockdown slightly upregulates Vg1
expression after 9 hours, but this effect is much less marked than the consequences on axis development.

Other candidate targets of GATA are likely to be members of the BMP family. To test the possibility that Gata2 may act through BMP, we examined the effects of Gata2/3-MO electroporation (within its normal anterior expression domain) on expression of Bmp4, 6 hours after cutting: 13/34 embryos (38%) showed downregulation of Bmp4 expression in the electroporated region (supplementary material Fig. S3C,D). By contrast, 0/30 embryos showed downregulation after control-MO electroporation (supplementary material Fig. S3E,F). Thus, the site of primitive streak formation may be determined by a balance between Vg1/Nodal-related (Smad2 activation) signals from the posterior margin and BMP-related (Smad1 activation), highest anteriorly and controlled indirectly by GATA, by regulation of BMP gene expression.

DISCUSSION

Our results suggest the existence of anterior cues that contribute to establish embryo polarity before gastrulation in the chick embryo. Previously, it was generally believed that the main, if not the only, cues for polarity reside posteriorly, the earliest one known to date being Vg1 (Seleiro et al., 1996; Shah et al., 1997). Here we show that GATA factors are expressed anteriorly even before the appearance of Vg1 in the posterior marginal zone. Surprisingly however, Gata2 downregulation in intact embryos does not immediately induce Vg1, nor does Vg1 misexpression immediately downregulate Gata2, suggesting that these opposing cues are under independent control. This raises the possibility that the entire embryo is patterned at a very early stage by instructions that almost simultaneously specify anterior (Gata2-expressing) and posterior (Vg1-expressing) parts of the marginal zone, as if there is an embryo-wide coordinate system specifying cell position (‘global positioning system’) responsible for localised expression of these factors at opposite ends of the embryo. The nature of the upstream regulators specifying the global coordinate system is unknown; the present study suggests that factors should be sought that can regulate gene expression throughout the embryo.

To date, chick embryos have been the main amniote model system for the study of embryonic regulation. In mouse, the earliest markers of embryonic polarity appear to be mainly localised posteriorly, but as it is impossible to predict the orientation of the embryo with accuracy before primitive streak formation, this is difficult to confirm (Tam and Behringer, 1997; Tam and Gad, 2004). Rabbit and other non-rodent mammals have been described as having a distinctive anterior region (‘anterior marginal crescent’, ‘anterior pregastrulation differentiation’ or ‘anti-sickle’) that can be defined morphologically (Viebahn et al., 1995; Hassoun et al., 2009), but no specific molecular components have yet been described as presaging the future axis and act as anterior determinants. It will be interesting to explore the expression of GATA factors in eutherian mammals to determine whether, as in the chick, they may represent very early markers of anterior (‘anti-gastrular’) position.

The non-cell-autonomous effects of Gata2-knockdown suggested the involvement of a secreted factor controlled by
Gata2: anterior specifier of embryo polarity

Although we observed a mild effect of GATA knockdown on expression of Vg1, this effect is much less pronounced than that on axis development. We therefore explored the BMP family. There is evidence that BMPs may lie both up- and downstream of Gata2. First, Gata2/3 genes can be downstream targets of BMP and downregulated by Smad2-dependent Activin/Nodal signals (Walmsley et al., 1994; Neave et al., 1995; Read et al., 1998). At the same time, GATA factors can lie upstream of BMPs, the expression of which they can upregulate (Sykes et al., 1998; Loose and Patient, 2004; Linker et al., 2009). There is also evidence that BMP activity can influence embryo polarity: misexpression of Bmp4 posteriorly in the chick blastoderm can suppress primitive streak formation, whereas the BMP antagonist Chordin can induce an ectopic primitive streak right up to the start of gastrulation (Streit et al., 1998; Streit and Stern, 1999). Our results suggest that Gata2 may act at least in part by modulating BMP.

It was recently argued that embryonic regulation in Xenopus is controlled by BMP-related signals at opposite poles (dorsal and ventral) of the embryo – dorsally the main signal is ADMP, whereas ventrally Bmp2, 4 and 7 predominate (Reversade and De Robertis, 2005). It was proposed that both dorsal and ventral poles are under some sort of global control, and that this is the result of BMP-related molecules acting over a considerable distance, based on the observation that depletion of Bmp2/4/7 ventrally causes upregulation of ADMP dorsally. However, it is important to point out that anuran amphibians are only capable of full embryonic regulation until the third cleavage division (the eight-cell stage), whereas in the chick the entire embryo can regenerate from any fragment isolated as late as the blastoderm (Eakin, 1999). BMP activity can influence embryo polarity: misexpression of Bmp4 posteriorly in the chick blastoderm can suppress primitive streak formation, whereas the BMP antagonist Chordin can induce an ectopic primitive streak right up to the start of gastrulation (Streit et al., 1998; Streit and Stern, 1999). Our results suggest that Gata2 may act at least in part by modulating BMP.


**Fig. S1. Gata3 is not expressed before primitive streak formation.** In situ hybridisation for Gata3 at pre-streak stages X (A) and XIII (B) show no expression, whereas embryos at stage 5‘ (C) show expression in the prospective epidermis, as previously reported (Sheng and Stern, 1999).

**Fig. S2. GATA knockdown does not induce or extend Koller’s sickle.** To test whether GATA knockdown could either induce an ectopic Koller’s sickle, or somehow attract sickle cells, Gata2/3 morpholinos were electroporated anteriorly (anti-fluorescein, brown) and embryos examined for ectopic expression of Chordin, which at this stage is specific for the sickle (purple). Neither ectopic expression nor an extension of the endogenous domain into the electroporated region was seen. The embryo shown is at stage XIII.
Fig. S3. Epistasis experiments to test whether GATA acts through induction of Vg1 or Bmp4. (A,B) GATA-MO was electroporated into the anterior half of an embryo (experimental design as in Fig. 4D), and expression of Vg1 examined after 9 hours. An example is shown after in situ hybridisation for Vg1 (A) and the same case after staining with anti-fluorescein to reveal the morpholino-electroporated cells (B). In this case, Vg1 is upregulated in the region where GATA was knocked down. (C-F) GATA-MO (C,D) or control-MO (E,F) was electroporated anteriorly in an intact embryo and Bmp4 expression examined 6 hours later. GATA-MO causes slight downregulation of Bmp4 expression (C,D) when compared with the control (E,F).