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Inhibition of FGF signaling causes expansion of the endoderm in *Xenopus*☆

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Abstract

Fibroblast growth factor (FGF) is established as an initiator of signaling events critical for neurogenesis and mesoderm formation during early *Xenopus* embryogenesis. However, less is known about the role FGF signaling plays in endoderm specification. Here, we show for the first time that endoderm-specific genes are induced when FGF signaling is blocked in animal cap explants. This block of FGF signaling is also responsible for a significant enhancement of endodermal gene expression in animal cap explants that are injected with a dominant-negative BMP-4 receptor (DNBR) RNA or treated with activin, however, neural and mesoderm gene expression is diminished. Consistent with these results, the injection of dominant-negative FGF receptor (DNFR) RNA expands endodermal cell fate boundaries while FGF treatment dramatically reduces endoderm in whole embryos. Taken together, these results indicate that inhibition of FGF signaling promotes endoderm formation, whereas the presence of active FGF signaling is necessary for neurogenesis/mesoderm formation.
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During early *Xenopus* embryogenesis, cells in the equatorial region of the embryo adopt a mesodermal fate in response to inductive signals emanating from the underlying vegetal hemisphere [1–4]. Vegetal cells secrete inductive signaling molecules that induce embryonic equatorial cells to adopt a mesodermal fate, while vegetal cells are specified as endoderm. This suggests the possibility that a similar set of inducing factors may be involved in the segregation of the mesendoderm during embryogenesis.

The extracellular signals responsible for mesendoderm formation have still not been clearly identified, but members of the TGF-β superfamily activated by VegT, a maternal vegetally localized T-box transcription factor, are known to induce the mesoderm as well as the endoderm [5–7]. It is also clear that fibroblast growth factor (FGF) secreted from the equatorial cells can act in concert with members of the TGF-β superfamily to induce ectoderm cells from a gastrula stage embryo to become mesoderm. For instance, mesoderm-specific genes, such as Xwnt8, Xhox3, Xbra, and Xnot, can be induced by FGF treatment in the animal cap explants. In contrast, expression of the truncated FGF receptor prevents the full spectrum of mesodermal genes from being induced by exogenous activin in the animal cap explants [8,9]. The injection of a truncated FGF receptor into whole embryos suppresses some mesoderm formation and results in embryos with reduced posterior structures, but relatively unaffected heads. While these data show that FGFs may play a critical role in TGF-β mediated mesoderm induction, it also provides support for the idea that inhibition of FGF signaling in vegetal cells may prevent these cells from adopting a mesodermal fate. The results of several studies have implicated

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many factors including VegT, Mixer, Xsox17α andβ, Xbic(Bix)s, Xnrs, Smad2, Vg1, and TGF-β as playing a role in endoderm formation [6,10–12]. Of particular interest, maternal VegT has been shown to initiate endoderm formation before the midblastula transition (MBT), but maintenance requires TGF-β signaling through cellular interactions [7]. Pre-requisite signaling for endoderm formation during early embryogenesis has not yet been fully addressed. In *Xenopus*, it was previously shown that high levels of both maternal XbFGF RNA and active FGF receptor 1 (XFGFR1) protein are present in animal cells relative to vegetal cells, and that FGF signaling inhibits the expression of an endoderm-specific gene, Xlhox8 [13]. FGF signaling is required for both induction of neural tissue via antagonism of the BMP-4 and for mesoderm formation in response to activin [9,14]. These studies led us to examine whether FGF signaling may be involved in germ layer specification.

In the present study, we employed dominant-negative FGF receptor (DNFR) [15] to examine whether cell fate was altered in ectodermal explants that would normally follow an epidermal cell fate if left untreated. Suppression of FGF signaling by DNFR elicited the induction of endoderm-specific genes in the animal cap explant assay. Endoderm gene expression was significantly enhanced by DNFR or SU5402 (a pharmacological inhibitor of FGFR1a [16]) in animal caps either expressing a dominant-negative BMP-4 receptor (DNBR) or treated with activin. Furthermore, in embryos, the over-expression of DNFR in the vegetal hemisphere resulted in expanded ventral structures and a shifting of the endoderm border into the animal hemisphere. These results indicate that embryonic cells adopt an endodermal fate when FGF signaling is abrogated and that the presence or absence of FGF signaling may be one of the critical factors responsible for the commitment to either a neuroectoderm/mesoderm or endoderm normally.

Materials and methods

Embryo manipulation. *Xenopus laevis* were obtained by in vitro fertilization [17]. Developmental stages were designated according to Nieuwkoop [18]. Embryos at the two-cell stage were injected into the animal pole or vegetal pole with mRNA or DNA as described in the figure legends. Animal caps were dissected from the injected embryos at stages 8.5–9 and cultured to various stages for further analysis as described in the figure legend.

DNA and RNA preparation. DNFR, a dominant-negative FGF receptor-1, was linearized by *Eco*RI and transcribed using SP6 polymerase. DNBR, a dominant-negative type I receptor for BMP-4, was transcribed according to previously described methods [17]. CSKA-eFGF was linearized with *Sac*I for a DNA injection. The CSKA vector is an expression vector containing a cytoskeleton actin promoter. Each linearized plasmid used for the in vitro synthesis of a capped mRNA using an mMessage mMachine kit (Ambion) was in accordance with the manufacturer's instructions. The synthetic mRNA was quantitated by ethidium bromide staining in comparison with a standard RNA marker (Gibco-BRL).

Tissue explant culture. Animal cap explants were dissected at stage 8.5–9 and cultured in normal salt media [30% Marc's modified Ringer (MMR) containing 50 μg/ml gentamicin] to various stages for further analysis as described in the figure legends. SU5402 (10 μM), a chemical inhibitor of FGFR1a, was used to block the FGF signal (Calbiochem). Activin (0.15 ng/ml) was used to induce mesoderm tissue in animal cap explants.

Tissue recombinants. Animal cap isolation and recombination was accomplished as described previously [19]. The conjugated recombinants were cultured for 40 min before transfer to 30% MMR media.

Reverse transcription-polymerase chain reaction. Total RNA was extracted from whole embryos or cultured explants with TRIzol reagents (Life Technologies) following the manufacturer's instructions. Reverse transcription-polymerase chain reaction (RT-PCR) was performed with a Superscript pre-amplification system (Invitrogen). Primers posted on Xenbase (http://www.xenbase.org) were used in RT-PCR.

Whole-mount in situ hybridization. Whole-mount in situ hybridization was performed as previously described [19], except BM purple AP substrate (Roche) was used and RNase treatment was omitted with *Albino* embryos. For in vitro synthesis, the Megascript kit (Ambion) was used following the manufacturer's instructions. The image was captured by a Nikon coolpix990 digital camera.

Results and discussion

Expression of DNFR causes endoderm formation in animal cap explants

FGF signaling is a critical event for mesoderm formation and neural induction in animal cap explant cultures [1]. To determine whether inhibition of FGF signaling alters embryonic cell fate, DNFR was expressed in embryos. In vitro transcribed DNFR RNA was injected at varying doses into the animal hemisphere at the two-cell stage. Injected embryos or animal cap explants were harvested and analyzed by RT-PCR and by in situ hybridization (ISH). The expression of an early and a late endoderm marker, *Mixer* [20] and endodermin (edd), was detected in the DNFR RNA-injected animal cap explants as shown in Fig. 1A. *Edd* is a late stage pan-endodermal marker, although it is also expressed in the dorsal mesoderm and endoderm in the early gastrula stage [21]. *Mixer* was induced in the animal cap explants from embryos injected with a low dosage of DNFR RNA (1 ng/embryo), but *edd* was detected only in the explants injected with higher amounts of DNFR RNA (5 ng/embryo) (Fig. 1A). These results suggest that the formation of later endodermal tissue may require a higher threshold level of DNFR and all *Mixer*-expressing tissue may not develop into later stage endoderm. The animal cap explants were also analyzed for general neural and mesodermal markers. Control RNA (β-galactosidase mRNA 5 ng) or DNFR RNA-injected animal cap explants did not contain any neural or mesoderm tissue, as evidenced by *N-CAM* and *muscle actin*, respectively (Fig. 1A). Treatment of animal caps

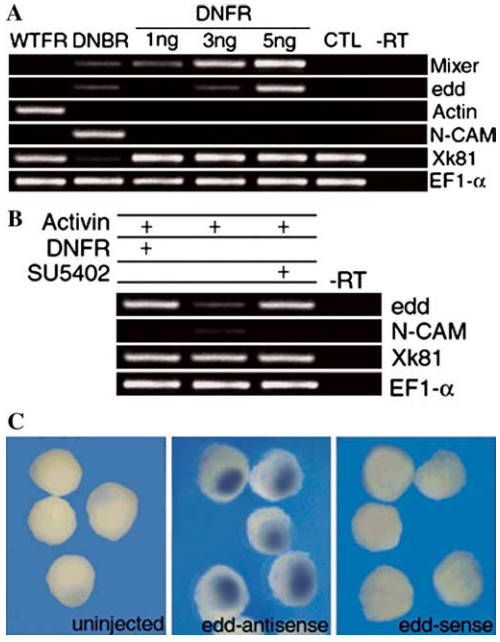

Fig. 1. (A) Truncated FGF receptor induces endoderm markers in animal cap explants. RT-PCR analysis of animal caps dissected at stage 8.5 and cultured until stages 11 and 24 to measure fate markers. *EF1-α* was used to normalize cDNA samples. The RT(−) lane contains all reagents except reverse transcriptase and is used as negative control. (A) *Mixer*, an early endodermal marker, and *edd* (endodermin), a late pan-endoderm marker, were induced by *DNFR* RNA in a dose-dependent manner. *Actin*, a mesoderm marker, was not induced. *N-CAM*, a late neural marker, was detected in dominant-negative BMP-4 receptor (*DNBR*)-injected animal cap. DNFR RNA (1–5 ng/embryo), wild-type FGF receptor (WTFR) (1 ng/embryo) RNA, and DNBR RNA (1 ng/embryo) were injected radially into each blastomere at two-cell stage. (B) Animal caps treated with SU5402, a chemical inhibitor of FGFR1a, also displayed endoderm marker induction in a dose-dependent fashion. (B) Induction of endoderm by *DNFR* expression or SU5402 treatment in activin-treated or DNBR RNA-injected animal caps. FGF signal inhibition (DNFR or SU5402) can enhance *edd* gene expression in the activin or DNBR-treated animal cap explants. DNFR or DNBR RNA-injected animal caps were dissected from embryos injected with 3 ng DNFR RNA or 1 ng DNBR RNA, respectively. Activin was added to a final concentration of 0.15 ng/ml (general mesoderm marker genes were induced at this concentration) and SU5402 was added to a final concentration of 10 μM. Animal caps were cultured until stage 24. (C) In situ hybridization using an *edd* probe. Left, uninjected explants hybridized with antisense *edd* probe. Center, an embryo injected with DNFR RNA. Strong expression of *edd* was detected when compared with sense probe treated explants (right). The explants in the center and right panels were derived from embryos injected with 3 ng DNFR RNA.

with the pharmacological inhibitor of FGFR, SU5402 [16], confirmed that endoderm formation was induced by blocking the FGF signaling (Fig. 1B).

ISH was performed in the *DNFR* RNA-injected animal cap explants to confirm the RT-PCR data. Expression analysis using ISH showed that *edd* was strongly induced in the DNFR-treated animal caps. Conversely, uninjected animal caps or animal cap explants hybridized with sense-strand control probes were not stained as shown in Fig. 1C and Table 1. Taken together, DNFR expression induced endoderm formation in animal cap explants, suggesting that FGF signaling may modulate endoderm specification during early *Xenopus* development.

Table 1
Edd expression in animal cap explants

	edd expressed	edd non-expressed
DNFR-injected animal cap [*n*(%)]	32(74)	11(26)
Control animal cap [*n*(%)]	0(0)	45(100)

Note. Explants were injected with DNFR (3 ng) or β-gal (3 ng) mRNA and analyzed by in situ hybridization at stage 24. The presented data are the result of two individual injection experiments.

Inhibition of FGF signaling enhances endoderm formation, but reduces neurogenesis and mesoderm formation induced by DNBR or activin, respectively

Since FGF signal inhibition alone caused endoderm formation in animal cap explant cultures, it was of interest to define the contribution of this signaling to neuroectoderm and mesoderm formation. Activin-induced mesoderm formation and neurogenesis resulting from expression of DNBR require FGF signaling in animal cap explants [9]. RT-PCR assays were performed with activin-treated animal caps either expressing DNFR or treated with SU5402. The expression pattern of endoderm markers was analyzed, and DNFR or SU5402 indeed enhanced *edd* gene expression even in activin-treated animal cap explants (Fig. 1B). In contrast, mesoderm formation was dramatically reduced as evidenced by inhibition of mesoderm marker expression (Fig. 1B). These results demonstrate that inhibition of FGF signaling commits ectodermal cells to an endoderm cell fate even in the presence of a mesoderm inducer.

In animal caps, blocking BMP-4 signal transduction via expression of DNBR induced both neural and endodermal markers [21]. Inhibiting FGF signaling by DNFR RNA-injection in addition to DNBR expression markedly enhanced endoderm marker expression (Fig. 1B). Furthermore, neural markers displayed a reduced level of expression due to the blocking of FGF signaling (Fig. 1B). These results suggest that FGF signal inhibition can induce endoderm cell fate specification while opposing mesoderm and neural inductive signals.

Confining DNFR expression to the animal and vegetal hemisphere elicits distinct phenotypes in whole embryos

Having established that blocking FGF signaling can induce endoderm in an ectodermal culture system where cell movement and juxtaposition of different tissues are restricted, we proceeded to examine whole embryos.

Varying doses of DNFR RNA were injected into either the animal or vegetal pole of two-cell stage embryos [1–5 ng (Fig. 2A and Table 2)]. When FGF signaling was blocked in either the vegetal or animal pole region, distinct morphological changes were observed. Embryos displayed shortened tails in a dose-dependent manner when DNFR was injected into the animal pole, supporting a previous report (Fig. 2A(a); [22]). In contrast, the embryos injected with *DNFR* RNA in the vegetal pole region presented only a small degree of tail truncation but a markedly expanded abdominal region (Fig. 2A(b)). In addition, eFGF RNA injection also led

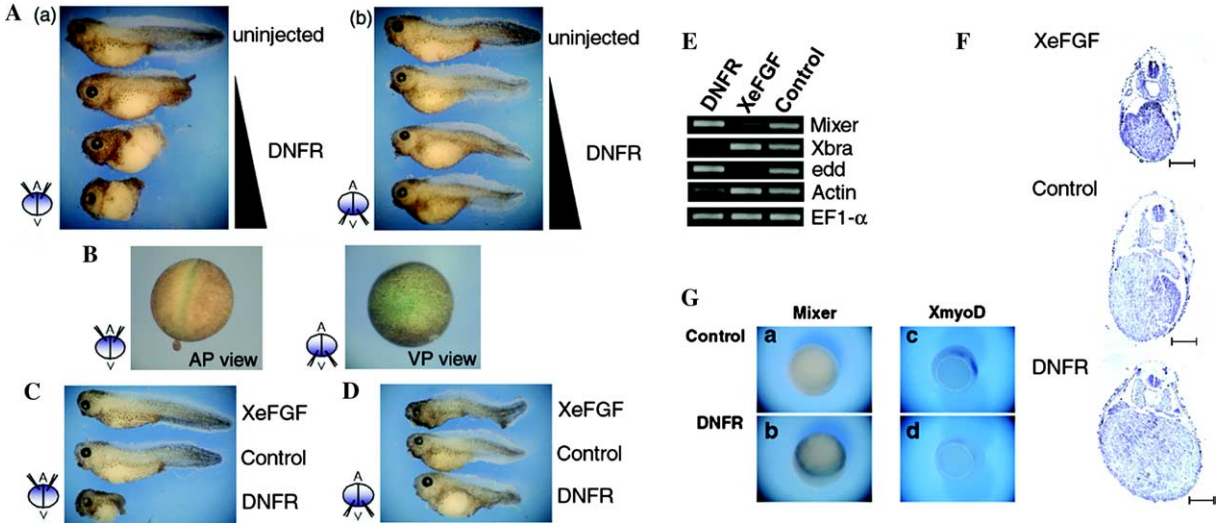

Fig. 2. (A) Dose-dependent phenotype from injection of DNFR RNA in animal hemisphere (a) or vegetal hemisphere (b). The injection of DNFR RNA injected at varying concentrations into two-cell stage embryos and cultured until stage 34. (a) Animal hemisphere injected groups show a reduction of posterior structures and open neural tube defects in a dose-dependent fashion consistent with a previous report [22]. (b) In the vegetally injected groups, the abdominal structure was expanded with increasing doses of DNFR RNA. In contrast, mesoderm and dorsal structures were not significantly affected in this group. (B) β-Gal lineage trace of vegetal or animal pole-injected embryos. As expected, X-Gal staining was observed in the notochord region of embryos when β-Gal mRNA was injected into the animal pole, while X-Gal staining was localized within the abdominal region upon vegetal pole injection. (C) DNFR or XeFGF RNAs were injected into the animal hemisphere at the two-cell stage. An extended tail was observed in the XeFGF-injected embryos. DNFR-expressing embryos lacked most posterior structures. (D) DNFR or XeFGF RNAs were injected into the vegetal hemisphere at the two-cell stage. General ventral structures disappeared in the XeFGF RNA-injected embryos, while the DNFR RNA-injected embryos had expanded ventral structures. (E) RT-PCR analysis. In the XeFGF RNA-injected embryos, mesoderm markers were up-regulated and endoderm markers were down-regulated, and the reverse was true of DNFR-expressing embryos. The results suggest that FGF signaling may play a role in the formation and maintenance of mesoderm and a barrier for excessive expansion of the endoderm. RT(−) lane was omitted. (F) Sections of embryos that underwent *DNFR*or *XeFGF* RNA injection into the vegetal hemisphere. As shown, endoderm tissue was greatly expanded when compared to control siblings. Paraffin-embedded sectioning was performed at a thickness of 10 μm at comparable depths. Scale bar: 100 μm (G). The expansion of endoderm in DNFR RNA-injected embryos. Whole-mount in situ hybridization of early gastrula (a and b) was performed using antisense *Mixer* probe as an endoderm marker. (a) Uninjected embryo. (b) DNFR RNA (3 ng/embryo)-injected embryos showed enlargement of endoderm tissue. Whole-mount in situ hybridization of early gastrula (c and d) was performed using antisense *XmyoD* probe as an early mesoderm marker. (c) Uninjected embryo. (d) The embryos injected with DNFR RNA (3 ng/embryo) exhibited diminished mesoderm tissue. The dashed circle indicates the boundary between sub-blastoporal endoderm and supra-blastoporal endoderm. (a–d; vegetal pole view).

Table 2
Abdominal expansion in DNFR-injected embryo

	DNFR mRNA		Control mRNA	
	Injected into AH	Injected into VH	Injected into AH	Injected into VH
Posterior structure missed [*n*(%)]	89(87)	15(16)	0(0)	0(0)
Abdominal structure expanded [*n*(%)]	0(0)	64(68)	0(0)	0(0)
General defect [*n*(%)]	3(3)	7(8)	0(0)	0(0)
Death [*n*(%)]	2(2)	3(3)	1(3)	0(0)
Normal [*n*(%)]	8(8)	5(5)	31(97)	42(100)
Total [*n*(%)]	102(100)	94(100)	32(100)	42(100)

Note. Embryos were injected with DNFR (1–5 ng) orβ-gal (5 ng) mRNA and analyzed by phenotypic observation at stages 31–34. The presented data are the result of two individual injection experiments. General defects include bent body and shortened anterior–posterior axis. AH, animal hemisphere; VH, vegetal hemisphere.

to morphologically distinct phenotypes that were dependent upon the localization of expression (Figs. 2C and D).

The morphological differences that are observed should result from the expression of DNFR in areas encompassing the specific sites of injection. To clarify this matter, β-Gal mRNA was injected into two groups of embryos in the same location as for DNFR RNA. X-Gal staining performed in the embryos at the neurula stage showed specific domains of expression (Fig. 2B). X-Gal staining was observed in the notochord region when β-Gal mRNA was injected into the animal pole, while the abdominal region was the site of expression in vegetal pole-injected embryos. These results are consistent with the morphological results that demonstrate a shortening of the A-P axis when FGF signaling is blocked in the animal pole-derived tissues and abdominal expansion in the vegetal cell progeny.

FGF signaling restricts the endoderm domain in embryos

In whole embryos, injection of DNFR RNA into the vegetal area caused the expansion of the abdomen region which is derived from endoderm progenitors. While this blockade of FGF signaling expands the endodermal structures, the activation of FGF signaling in the vegetal region by an eFGF RNA injection caused the reduction of these structures (Fig. 2D). To confirm the morphological results, a histological study was performed in the embryos injected with DNFR or eFGF RNAs after paraffin-embedding (Fig. 2F). As expected, sections of the *DNFR* RNA-injected embryos showed increased endodermal tissue in the abdominal region while the endodermal mass was reduced significantly in the eFGF over-expressing embryos. This confirms that endoderm formation can be regulated by the presence and absence of FGF signaling in whole embryos as well as in animal cap explants.

The molecular markers for the endoderm and mesoderm were examined in whole embryos by RT-PCR analysis (Fig. 4E). As expected, the mesodermal markers, Xbra and actin, were down-regulated while the endodermal markers, *Mixer* and *edd*, were up-regulated in the DNFR-expressing embryos. The same kind of molecular markers was examined in the eFGF RNA-injected embryos and the opposite expression pattern was observed, supporting the morphological and histological results.

Since the morphological and molecular changes were observed in the later stage embryos, we examined the changing pattern of endodermal mass resulting from DNFR or eFGF expression in early stage embryos. A whole-mount in situ hybridization (WISH) was performed to visualize the expression pattern of the mesendodermal markers. As expected, the expression of the early endodermal marker, *Mixer*, was expanded while that of the mesodermal marker, XmyoD, was dramatically reduced when DNFR RNA was injected into the vegetal region of the embryo. The WISH data also indicated that DNFR increased the boundaries of the endoderm region, and contracted in the mesoderm domain in the early stage embryos (Fig. 2G). These results suggested that FGF may not only be acting as an essential element for mesoderm induction and neuroectoderm formation, but also functioning as a barrier to restrict the expansion of endoderm in the equatorial region of early embryos. These data further support the idea that the presence or absence of FGF signaling is crucial for mesendoderm specification.

Animal cells expressing DNFR induce endoderm but are not competent to induce mesoderm

Vegetal cells have been known to develop into endodermal tissue and, when combined with animal caps, induce mesoderm [3,18]. The tissue recombination technique was applied to investigate whether animal cap explants with FGF signaling inhibited could mimic the vegetal cells' ability to induce mesoderm. The recombinants were generated by the conjugation of *DNFR* RNA-injected animal caps and uninjected ones (Fig. 3A). The resulting recombinant tissues were examined for the expression of endodermal and mesodermal markers. *DNFR*-expressing recombinants induced early and late endodermal genes, *Mixer* and *edd*, respectively, as expected (Fig. 1A). The recombinants, however, did not induce the expression of the early mesoderm marker, *Xbra*, nor the later mesoderm markers, *actin* and *globin*. It is interesting to note that the expression of the early ventral mesoderm gene,

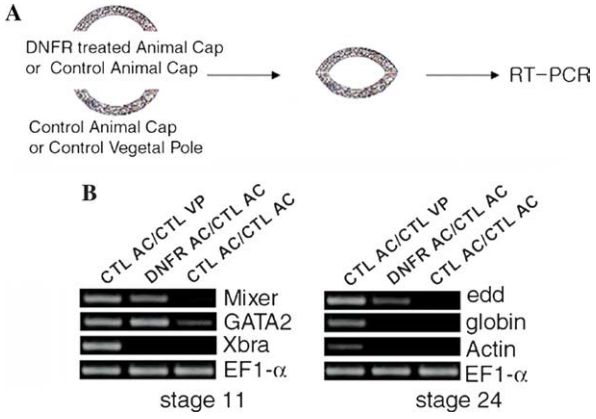

Fig. 3. Blocking FGF signaling in animal cap explants induced endoderm that lacked mesoderm inducing activity. (A) Tissue recombination technique. (B) DNFR-expressing recombinants (DNFR animal cap/control animal cap) also expressed early and later endoderm genes, *Mixer* and *edd*, as expected. However, the recombinants did not induce the early mesoderm marker, *Xbra*, nor the late mesoderm markers, *actin* and *globin*. An early ventral mesoderm marker (*GATA2*) was detected in the DNFR animal cap/control animal cap recombinants. This result indicates that the absence of FGF signaling in animal cells generates endoderm, but it is not competent to induce mesoderm in adjacent tissue. RT(−) lane was omitted.

GATA2, was increased in *DNFR* RNA-injected recombinants (Fig. 3B). These results showed that blocking FGF via *DNFR* led to endoderm development in animal cap explants, however, this tissue failed to induce mesoderm in adjacent cells. Thus, the absence of FGF signaling in animal cells promotes endoderm, but it is not competent to induce mesoderm [5,6,23–26].

FGF signaling may regulate the endoderm/mesoderm boundary

A two-step model in endoderm formation has been proposed in *Xenopus* [11,27]. VegT promotes the initial step in this model. Further studies prove that VegT can induce a set of transcription factors like Sox17s, Mix1, Xnrs, and Derriere [6], which maintain a VegT expression level and also activate endoderm-inducible transcription factors like *Mixer* and Xnrs [28]. These endoderm-inducible factors then influence the marginal zone regarding mesoderm formation by restricting or defining the border between endoderm and mesoderm. In *Xenopus*, the two early endodermal determinants which have been isolated to date, VegT and Vg1, regulate the induction of the mesoderm and endoderm. Vg1, the zygotic Nodal-related molecule, is a member of the TGF-β superfamily and is involved in the formation of the mesoderm and endoderm as demonstrated by gain- or loss-of-function studies [24,29]. VegT, one of the several T-box transcription factors, was shown to be an endoderm inducer as a result of loss-of-function [5]. Using antisense oligonucleotides showed that maternal VegT is required for 90% of mesoderm formation, as well as endoderm initiation [25]. These results suggest that an additional factor may exist to discriminate endoderm from mesoderm formation in the vegetal hemisphere.

The level of FGFR translation in the presumptive endoderm is lower than that in the presumptive mesoderm, and eFGF, a ligand, is also expressed mainly in the marginal zone. Therefore, it seems that FGF signaling may be highly active in the presumptive mesoderm while being less active in the endoderm. Furthermore, eFGF, FGF-3, and FGF-8 showed a relatively poor ability to rescue the phenotype of VegT-depleted embryos [25]. These data appear contrary to a role for FGF signal transduction in the commitment to an endodermal fate, since one might assume that vegetal cells may display a lack of FGF signaling, a similar state as the DNFR-expressing group in our study. In contrast, FGF has previously been proposed to be an important factor in the commitment to mesoderm or endoderm, although the response of endoderm-specific genes to FGF signaling was not reported [9].

To explain these seemingly disparate results we propose a modification of previously presented models for mesendoderm formation in *Xenopus*, as shown in Fig. 4.

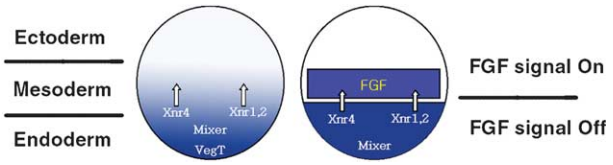

Fig. 4. The proposed model: the role of FGF signaling as a regulatory factor in mesendoderm formation. In the two-step model of mesendoderm formation [5,11], maternal factors like VegT induce TGF-β-related signaling molecules and these molecules subsequently induce mesendoderm. A modified model is proposed to explain how similar signals can induce two different germ layers and factor(s) that differentiate these two germ layers. In the proposed model, the spatially restricted existence of FGF signaling in the equatorial region acts as a natural barrier to prevent this area from succumbing to a endoderm fate in early embryogenesis. The absence of FGF signaling in the vegetal area is proposed to allow the proper microenvironment for endoderm formation driven by signals within the vegetal cells. In contrast, FGF signaling in the equatorial region is a competence factor for adjacent mesoderm formation in response to vegetal cell signals.

This model suggests that FGF signaling may be functioning as an inhibitory factor that restricts the expansion of endoderm in the equatorial region in response to endoderm induction factors in the vegetal hemisphere. Thus, FGF signaling in the equatorial region may be a competence factor for mesoderm formation that is induced by signals emanating from vegetal cells, while the absence of FGF signaling in the vegetal hemisphere may provide the proper microenvironment for endoderm induction. In summary, we propose that the spatially restricted existence of FGF signaling in the equatorial region acts as a natural barrier to delimit the territory of endoderm induction during early embryogenesis.

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