mRNA localization studies suggest that murine FGF-5 plays a role in gastrulation

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Summary

During gastrulation in the mouse, the pluripotent embryonic ectoderm cells form the three primary germ layers, ectoderm, mesoderm and endoderm. Little is known about the mechanisms responsible for these processes, but evidence from previous studies in amphibians, as well as expression studies in mammals, suggest that signalling molecules of the Fibroblast Growth Factor (FGF) family may play a role in gastrulation. To determine whether this might be the case for FGF-5 in the mouse embryo, we carried out RNA in situ hybridization studies to determine when and where in the early postimplantation embryo the Fgf-5 gene is expressed. We chose to study this particular member of the FGF gene family because we had previously observed that its pattern of expression in cultures of teratocarcinoma cell aggregates is consistent with the proposal that Fgf-5 plays a role in gastrulation in vivo. The results reported here show that Fgf-5 expression increases dramatically in the pluripotent embryonic ectoderm just prior to gastrulation, is restricted to the cells forming the three primary germ layers during gastrulation, and is not detectable in any cells in the embryo once formation of the primary germ layers is virtually complete. Based on this provocative expression pattern and in light of what is known about the functions in vitro of other members of the FGF family, we hypothesize that in the mouse embryo Fgf-5 functions in an autocrine manner to stimulate the mobility of the cells that contribute to the embryonic germ layers or to render them competent to respond to other inductive or positional signals.

Key words: gastrulation, mouse embryogenesis, Fgf-5, fibroblast growth factor-5.

Introduction

Gastrulation is generally defined as the process whereby a population of embryonic cells is transformed into a three-layered structure, consisting of ectoderm, endoderm and a middle layer of mesoderm. In mammals, the starting cell population, known as the embryonic ectoderm or epiblast, can be thought of as a flat, roughly circular, pseudostratified, columnar epithelial sheet. At some time prior to the onset of gastrulation, a particular region on the sheet's circumference becomes determined as the posterior end of the embryo, and a corresponding position ~180° around the circumference becomes defined as the anterior end. Gastrulation begins when cells within the sheet move towards the posterior end of the embryo. This presumably occurs by a process of movement within the epiblast, rather than by cells exiting from the sheet and moving across its surface (Lawson et al. in preparation). The net effect of this movement is observed as an accumulation of cells at the posterior end of the sheet. This thickening in the cell sheet is known as the primitive streak. As gastrulation proceeds, cell movement within the sheet results in progressive accumulation of cells (lengthening of the primitive streak) along a line from the circumference of the sheet towards its center. Following their accumulation in the streak, cells exit from it and go on to form the mesodermal and endodermal cell layers.

Little is known about the key steps in this process, such as what regulates the movement of cells within the sheet, how this movement results in cell accumulation at the posterior end, or how cells acquire either a mesodermal or an endodermal cell fate. Similarly, there is little information about the molecules that regulate and mediate these processes. Our goal is to identify such molecules. Our approach is based on the widely accepted hypothesis that basic mechanisms of vertebrate embryogenesis have been evolutionarily conserved, and therefore that specific molecules that play a key role in gastrulation in other organisms also do so in mammals.

Based on this hypothesis, members of the FGF gene family are good candidates for molecules that play some role in gastrulation. They are known to induce gastrulation-like movements in isolated animal caps from Xenopus blastulae (Paterno et al. 1989; reviewed...
by Smith, 1989). In addition, of the seven known members of this family (Goldfarb, 1990), at least four have the capacity to induce amphibian embryonic cells that would otherwise assume an ectodermal fate to differentiate as mesoderm (Kimmel and Kirschner, 1987; Slack et al. 1987; Slack et al. 1988; Paterno et al. 1989). We previously cloned the mouse cognates of four of these genes and provided a preliminary description of their patterns of expression in the developing mouse embryo (Hébert et al. 1990). Based on those data, we proposed that Fgf-5 is likely to play a role during early postimplantation development. To explore the possibility that it has a function in gastrulation, we have carried out RNA in situ hybridization analyses of gastrulating embryos. The results obtained demonstrate that although Fgf-5 mRNA is not detectable in the mouse embryo shortly after implantation, expression increases dramatically just prior to the onset of gastrulation in the epithelial cells that give rise to the three germ layers. Throughout gastrulation Fgf-5 expression appears to be restricted to the cells that are forming the three primary germ layers, and by the time germ layer formation is virtually complete, Fgf-5 mRNA is undetectable. This unique pattern of expression, especially its temporal restriction to the period when cells within the epithelium are mobile, suggests to us that the FGF-5 protein may participate in the maintenance of the mobile state. Alternatively, since Fgf-5 expression ceases shortly after cells become allocated to particular lineages, another possibility is that FGF-5 may play a role in sustaining the pluripotency of the cells that form the germ layers, and thus renders them competent to respond to inductive or positional signals.

Materials and methods

Embryos and teratocarcinoma cell cultures

Mouse embryos at various stages of development were obtained by mating random bred ICR animals (Simenson Laboratories, Gilroy, CA). Embryonic age was estimated by taking the afternoon of the day on which the copulation plug was detected as 0.5 days of gestation (E0.5). There was, however, considerable variation in developmental stage both between and within litters at a given embryonic age. Embryos at the designated stages were dissected from the implantation site and fixed in 4% paraformaldehyde.

The origin of the PSA-1 teratocarcinoma stem cell line and the methods used to obtain differentiation (embryoid body formation) were exactly as described by Martin et al. (1977). At various times after differentiation had begun, PSA-1 cell aggregates were collected from suspension culture and fixed in 4% paraformaldehyde.

RNA probes

A plasmid clone containing the full-length Fgf-5 coding sequence (Hébert et al. 1990) was linearized with EcoRV before being transcribed with T7 polymerase to generate a α32P-UTP (1400 Ci mmol-1, New England Nuclear)-labeled single-stranded antisense RNA probe. Alkaline hydrolysis was used to reduce probes to an average size of 50–150 nt (Cox et al. 1984). The specificity of this probe for Fgf-5 mRNA is evidenced by the fact that the in situ hybridization pattern observed does not coincide with that obtained using probes for two other members of the FGF gene family, Fgf-k (Niswander and Martin, unpublished data) and int-2 (Wilkinson et al. 1988). Furthermore, a DNA probe composed of the same Fgf-3 sequence does not cross-hybridize with mRNA from any of the other known FGF family members in northern blot analyses at moderate stringency; the only exception to this was in one unusual experiment in which faint cross-hybridization with Fgf-k mRNA was observed (Hébert et al. 1990). When a single-stranded sense RNA probe was used as a control, no specific hybridization was observed (data not shown).

In situ hybridization

Embryo and teratocarcinoma cell samples were dehydrated, embedded in paraffin wax and sectioned (6 μm). In situ hybridizations were performed at high stringency as described by Frohman et al. (1990). The probe concentrations used in the hybridization solution ranged from 1 to 5×106 cts min-1 μl-1. Slides were exposed for one to two weeks before being developed and stained.

Results

Expression of Fgf-5 in the gastrulating mouse embryo

To determine the pattern of Fgf-5 mRNA expression during gastrulation, we carried out RNA in situ hybridization studies of mouse embryos at various stages of early postimplantation development. In the period between implantation and the start of gastrulation (~E4.5–E6.5; Fig. 1A, D), the embryonic portion of the conceptus develops from a solid ball of rounded cells into a cup-shaped epithelial cell sheet, the epiblast, surrounding a space known as the proamniotic cavity. The apical surface of the epiblast faces the cavity. Its basal (outer) surface is covered by a single layer of cells known as the visceral endoderm. These cells are not destined to form part of the fetus, but rather will contribute to extraembryonic membranes (Gardner, 1982; Gardner, 1984). The ‘base’ of the cup is the distal end of the embryo, and the ‘rim’ is its proximal end. Abutting the proximal end of the embryo is extraembryonic tissue, through which the embryo is connected to the uterine wall (Fig. 1A, D).

In one of the embryos removed from the uterus during this period (~E5.5), it appeared that the proamniotic cavity was just forming and the embryonic ectoderm had not yet become organized into an epithelial sheet. In that embryo, Fgf-5 mRNA was not detectable (Fig. 1B, C). In embryos at a slightly more advanced stage of development (E6.0–E6.5), Fgf-5 mRNA was readily detectable at uniformly high levels throughout the embryonic ectoderm (Fig. 1E, F, and data not shown). It was clear from analysis of serial sections that in some of these embryos the primitive streak had not yet formed (data not shown). In contrast, Fgf-5 mRNA was undetectable in the proximal extraembryonic tissue. The boundary between the Fgf-5-expressing and non-expressing tissues was very sharp. With respect to the visceral endoderm cells, which surround the embryonic ectoderm but will
ultimately form extraembryonic tissue, it was difficult to determine whether Fgf-5 mRNA was expressed in them since they are extremely flat and closely apposed to the strongly positive embryonic ectoderm (Fig. 1E, F).

A few hours later (the early-streak stage, ~E6.5), gastrulation begins as cells within the epiblast begin to move towards and accumulate in the primitive streak (Fig. 2A). As gastrulation proceeds (early- to mid-streak stage, ~E6.5–E7.0), cells that have accumulated in the streak begin to exit from it. Some of the cells that become mesoderm move proximally and cross the boundary between the epiblast and the extraembryonic region, where they develop into the extraembryonic mesoderm. The remainder of the mesodermal cells spread laterally towards the anterior end of the embryo, between the epiblast basal surface and the visceral endoderm (see Fig. 2B). Cells that become embryonic endoderm incorporate into and replace the outer endodermal cell layer. Finally, cells that remain in the epiblast are the precursors of the neuroectoderm and epidermis (Lawson and Pedersen, 1987; Tam, 1989; Carey et al. in preparation; Lawson et al. in preparation).

At this stage (~E7.0), expression of Fgf-5 remained restricted to the embryonic portion of the conceptus (Fig. 2C, D, and data not shown). Levels of Fgf-5 mRNA appeared to be relatively high throughout the embryonic ectoderm and primitive streak. There was, however, some evidence of differential expression along the proximodistal axis in the embryonic ectoderm: based on estimates of grain counts, it appeared that the signal intensity in the most proximal region was 2- to 3-fold lower than in the most distal region.

Fgf-5 mRNA was not detected in the mesodermal cells that had migrated proximally into the extraembryonic region, nor was it observed in those mesodermal cells that had migrated proximolaterally between the embryonic ectoderm and visceral endoderm (lateral...
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Endoderm Embryonic Ectoderm mesoderm). It is possible that these mesodermal cells are derived from embryonic ectoderm cells that never expressed Fgf-5. However, we consider this possibility unlikely, since the fate of the majority of the cells in the proximal posterior region of the embryonic ectoderm at the pre-streak stage is to form extraembryonic mesoderm (Lawson et al. in preparation), and we observed high levels of Fgf-5 expression in those cells. By contrast, embryonic mesodermal cells that have exited the streak in the more distal region of the embryo (paraxial mesoderm) did appear to contain Fgf-5 mRNA at levels similar to those observed in the distal embryonic ectoderm.

Some of the cells that exit the primitive streak at the early- to mid-streak stage assume a definitive endodermal cell fate. They insert into the endodermal cell layer and mix with the visceral endodermal cells of which it was initially composed (Lawson and Pedersen, 1987). We observed both Fgf-5-expressing and non-expressing cells in the endodermal cell layer (Fig. 2C, D). In cells covering the distal portion of the embryo, Fgf-5 mRNA was detected at levels equal to or higher than those in the adjacent embryonic ectoderm whereas, in cells covering the more proximal region of the embryo and in the extraembryonic endoderm, no Fgf-5 mRNA was detectable. The boundary between the positive and negative cells was extremely sharp, and the change in expression pattern appeared to correlate with a change in endodermal cell morphology. Thus, it seems likely that the Fgf-5-positive cells in the endodermal cell layer are definitive endodermal cells that are continuing to express Fgf-5 after they have exited from the primitive streak, rather than visceral endodermal cells.

During the mid- to late-streak stage (Fig. 3A), the primitive streak increases in length until it stretches from the proximal boundary (i.e. the rim of the cup) to the distal tip of the embryo. Just anterior to the distal end of the primitive streak, cells insert into the outer
Fig. 3. Expression of Fgf-5 in the mouse embryo at the late-streak and early neurula stages of development. (A) Diagram of the embryo at the late-streak stage, showing almost complete anterior migration of the embryonic mesoderm. The plane through which the embryo shown in B and C was sectioned is shown. (B) Hybridization of the antisense Fgf-5 probe to a near-sagittal section of an embryo (~E7.5) at the late-streak stage, showing the head process. (C) Same, dark field. (D) Hybridization of the antisense Fgf-5 probe to a near-frontal section of an embryo (~E7.75) in which the mesoderm completely surrounds the embryonic ectoderm and neurulation has begun, as evidenced by the appearance of the head folds. This embryo was a littermate of the one shown in B and C, and was embedded adjacent to it in the paraffin block; both embryos were therefore subjected to the in situ hybridization protocol under identical conditions. (E) Same, dark field. Abbreviations: Extraembryonic tissues: al, allantois; am, amnion; ve, visceral endoderm. Embryonic tissues: ec, embryonic ectoderm; de, definitive endoderm; hf, head folds; hp, head process; m, embryonic mesoderm. Magnification=×200.

endodermal layer and thus form the exposed 'head process,' which subsequently extends anteriorly in the midline and contributes to the notochord, trunk endoderm and other structures (Poelmann, 1981; Lawson et al. 1986; Lamers et al. 1987).

At this stage (~E7.5), embryonic ectoderm continues to express Fgf-5 mRNA, with increasingly higher levels detectable towards the distal end. No expression was detected in the extraembryonic or proximolateral embryonic mesoderm, whereas the distal embryonic mesoderm was positive and contained roughly the same levels of mRNA as were observed in the adjacent ectoderm. The endoderm surrounding the embryo, which is still a mixed population of epiblast-derived and primitive endoderm-derived cells (Lawson et al. 1986), contains levels of Fgf-5 mRNA similar to those in adjacent mesoderm. The head process cells are also positive, containing the highest levels of Fgf-5 mRNA detected in the embryo at this stage (Fig. 3B, C, and data not shown).

Shortly after the late-streak stage (E7.5–7.75), the embryo expands most markedly in the posterior-anterior direction, and thus is transformed into a more open bowl-shaped structure. Neurulation begins, as evidenced by the appearance of the head folds of the neuroectoderm (Fig. 3D). Our most striking observation was that Fgf-5 mRNA was no longer detectable in embryos that had reached this stage (~E7.75), as
determined by analysis of serial sections through the embryos (Fig. 3D, E, and data not shown), although it was abundant in embryos from the same litter that were not as advanced in development (Fig. 3B, C, and data not shown). Thus it appears that expression abruptly ceases when mesoderm has completely covered the basal surface of the embryonic ectoderm and formation of the three primary germ layers is virtually complete. Fgf-5 transcripts were also not detected in embryos at ~E8.0 (data not shown), although they are detectable in specific tissues at later stages of development (Niswander and Martin, unpublished data) as well as in the adult (Haub et al. 1990).

Expression of Fgf-5 in teratocarcinoma cell cultures
Certain teratocarcinoma stem cell lines, including the PSA-1 cell line, differentiate in vitro in a manner that is remarkably similar to the development of the early postimplantation mouse embryo (Martin, 1980). The steps in this process, which is known as embryoid body formation, include: (1) the formation of a layer of extraembryonic endoderm surrounding a pluripotent cell core that is analogous to the embryonic ectoderm; (2) the formation of a cavity within the core, which is analogous to the proamniotic cavity; (3) the reorganization of core cells into a columnar epithelium resembling the pre-streak epiblast; (4) the expansion of the embryoid bodies with concomitant transformation of the pluripotent core cells into mesoderm-like cells similar to extraembryonic mesoderm (Martin et al. 1977). Since these embryoid bodies can be produced in large quantities under controlled conditions in vitro, they provide a more readily accessible source of material for genetic and biochemical manipulations than do embryos at the equivalent stages of gestation.

We had previously found by northern blot analysis that there is a dramatic (~15-fold) increase in steady-state levels of Fgf-5 mRNA when PSA-1 cells form embryoid bodies (Hébert et al. 1990). We were therefore interested in determining which cells in the embryoid bodies express Fgf-5, and whether the pattern of expression is analogous to that in the gastrulating embryo.

Shortly after formation of the outer endodermal cell layer, relatively low levels of Fgf-5 mRNA were detected in most or all cells in the cultures. It is difficult to determine whether this is due to non-specific hybridization, or if it reflects a low level of expression in the embryoid bodies. We think that the latter is more likely, since our previous studies showed that Fgf-5 mRNA was detectable by northern blot hybridization in cells at that stage (Hébert et al. 1990). Once cavitation had begun, high levels of Fgf-5 mRNA were detected in ~20% of the embryoid bodies, and these levels appeared to be higher when the embryoid bodies had been kept in suspension culture for a longer period of time. In all cases, this high level of expression was observed only in the core cells of cavitated embryoid bodies, but not in their endodermal or mesodermal derivatives (Fig. 4), which are analogous to extra-embryonic cells in the normal embryo.

These data suggest to us that Fgf-5 expression is regulated in embryoid bodies in the same way as it is in the early gastrulating embryo: it is expressed at high levels as embryonic ectoderm cells progress beyond the cavitation stage and is down-regulated when the cells form mesoderm. We presume that the reason such abundant Fgf-5 mRNA expression is detected in the core cells of only ~20% of embryoid bodies examined at any particular time, is that in an asynchronously developing population of embryoid bodies only a fraction of them contains cells that have reached a stage appropriate for Fgf-5 expression and have not yet progressed beyond it. Moreover, these results suggest to us that cultures of PSA-1 teratocarcinoma cells can serve as an in vitro model system for studying the regulation of Fgf-5 gene expression as well as its function in the embryo.

Discussion
The data reported here describe the pattern of Fgf-5 expression in the early postimplantation mouse embryo. What is most striking about this expression is that it commences shortly before the onset of gastrulation and abruptly ceases when formation of the three primary germ layers is virtually complete; moreover, it is limited to the cells that are giving rise to the three primary germ layers. This suggests that Fgf-5 has some function in gastrulation. In trying to formulate a hypothesis about what this function might be, we have taken into consideration what is known about the specific processes occurring in the embryo during gastrulation and about the biological activities of the different members of the FGF family.

As previously described, cell mobility within the epiblast is a key feature of gastrulation. Although there is little direct evidence concerning patterns of cell movement in the mammalian epiblast (Daniel and Olson, 1966), it has recently been shown that the daughters of epiblast cells labelled at the pre- and early-streak stages are later found widely separated from one another (Lawson et al. in preparation), whereas the daughters of cells labelled at the late-streak stage are subsequently found adjacent to one another (Carey et al. in preparation). This suggests that there is considerable cell mobility within the epiblast early in gastrulation and little if any by the late-streak stage. Thus, there is a good correlation between the period of cell mobility within the epiblast and the time during which Fgf-5 is expressed in that tissue.

One possibility is that FGF-5 is required to sustain cell mobility within the embryonic ectoderm during gastrulation. Since FGF-5 is known to be secreted (Haub et al. 1990), its activity in this case could be autocrine. The observation that the level of Fgf-5 mRNA is reduced in the proximal region of the embryonic ectoderm by the mid-streak stage would be consistent with this hypothesis if cell mobility decreases earlier in that region than in the more distal part of the embryo. Such a function for FGF-5 is consistent with
the observation that the release of a related protein, basic FGF, from bovine aortic endothelial cells is required for their migration (Sato and Rifkin, 1988; Tsuboi et al. 1990).

After cells exit the streak, they appear to cease expressing Fgf-5 mRNA. There are two possible explanations for the observed differences in levels of Fgf-5 expression in cells that have passed through the streak (high in definitive endodermal cells and embryonic mesodermal cells exiting from the more anterior [distal] portions of the streak and undetectable in mesodermal cells that have exited from the posterior end). One is that Fgf-5 expression persists for different lengths of time in different cell populations; alternatively, it may be down-regulated at a particular time after exit from the streak and the cells that we found to contain Fgf-5 mRNA are those that have most recently emerged from the streak. The finding that the highest levels of Fgf-5 mRNA at the late-streak stage are in the emerging head process is consistent with the latter idea.

The expression of one other member of the FGF gene family, int-2, has been studied in the gastrulating mouse embryo (Wilkinson et al. 1988). It is evident from a comparison of the data from that study and the results described here that there is some overlap in the domains of expression of int-2 and Fgf-5, since both are expressed in the primitive streak. However, int-2 continues to be expressed in mesodermal cells migrating proximally to the extraembryonic region, whereas Fgf-5 expression appears to be down-regulated as cells leave the primitive streak and is never detected in the extraembryonic mesoderm. In addition, Fgf-5 mRNA is detected throughout the embryonic ectoderm, whereas int-2 mRNA is not. Furthermore, whereas int-2 expression persists in the primitive streak until E9.5, Fgf-5 expression abruptly ceases at the start of neurulation. Based on their observations, Wilkinson et al. (1988) suggested that int-2 functions to stimulate the migration of proximal embryonic and extraembryonic mesodermal cells. Thus it is intriguing to speculate that INT-2 and FGF-5 may have related functions in different cell types in the gastrulating embryo.

Another key feature of gastrulation is that during this process lineage decisions are made. By the late streak/head fold stages (−E7.75), most of the cells that are destined to form mesoderm or endoderm have already entered the primitive streak, whereas those that will become neuroectoderm or epidermis remain in the embryonic ectoderm (Tam, 1989; Carey et al. in preparation). It is possible that cells that are allocated
in this way have become committed to their fates. In that case, the temporal pattern of Fgf-5 expression would correlate with the period of pluripotency in the epiblast. Thus, another possible role of FGF-5 may be to render cells in the embryonic ectoderm competent to respond to signals that specify positional values or cell lineages. Basic FGF is thought to have such a function in the development of a neural crest cell lineage: it appears to render chromaffin cells competent to respond to nerve growth factor, which is needed for their differentiation into sympathetic neurons (Stemple et al. 1988; Birren and Anderson, 1990).

The two hypotheses about the possible function of FGF-5 proposed here are not inconsistent with the notion that FGFs play a role in mesoderm formation in *Xenopus* (reviewed by Smith, 1989). FGF-5 could have an indirect role in this process by stimulating the mobility of epiblast cells or by rendering them competent to respond to inductive or positional signals. Alternatively, in view of its known activity as a mitogen for cultured fibroblasts (Zhan et al. 1988), FGF-5 may function to stimulate the proliferation of cells during gastrulation. However, if this were the case, it is unclear why Fgf-5 expression ceases abruptly at the beginning of neurulation whereas cell proliferation continues.

It is obvious that considerably more information is required before we can go beyond mere speculation about the possible function(s) of Fgf-5 during gastrulation. Experiments are in progress to determine the temporal and spatial patterns of expression of the FGF-5 protein, as well as the cell surface receptor(s) through which it presumably acts, and to determine the effects on development of eliminating Fgf-5 gene expression. The results of such studies should provide us with a clearer picture of what the function of Fgf-5 might be, as well as the mechanism underlying a process in gastrulation.

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