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Low proliferative and high migratory activity in the area of *Brachyury* expressing mesoderm progenitor cells in the gastrulating rabbit embryo

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SUMMARY

General mechanisms initiating the gastrulation process in early animal development are still elusive, not least because embryonic morphology differs widely among species. The rabbit embryo is revived here as a model to study vertebrate gastrulation, because its relatively simple morphology at the appropriate stages makes interspecific differences and similarities particularly obvious between mammals and birds. Three approaches that centre on mesoderm specification as a key event at the start of gastrulation were chosen.

(1) A cDNA fragment encoding 212 amino acids of the rabbit *Brachyury* gene was cloned by RT-PCR and used as a molecular marker for mesoderm progenitors. Whole-mount in situ hybridisation revealed single *Brachyury*-expressing cells in the epiblast at 6.2 days post conception, i.e. several hours before the first ingressing mesoderm cells can be detected histologically. With the anterior marginal crescent as a landmark, these mesoderm progenitors are shown to lie in a posterior quadrant of the embryonic disc, which we call the posterior gastrula extension (PGE), for reasons established during the following functional analysis.

(2) Vital dye (DiI) labelling in vitro suggests that epiblast cells arrive in the PGE from anterior parts of the embryonic disc and then move within this area in a complex pattern of posterior, centripetal and anterior directions to form the primitive streak.

(3) BrdU labelling shows that proliferation is reduced in the PGE, while the remaining anterior part of the embryonic disc contains several areas of increased proliferation. These results reveal similarities with the chick with respect to *Brachyury* expression and cellular migration. They differ, however, in that local differences in proliferation are not seen in the pre-streak avian embryo. Rather, rabbit epiblast cells start mesoderm differentiation in a way similar to *Drosophila*, where a transient downregulation of proliferation initiates mesoderm differentiation and, hence, gastrulation.

Key words: Primitive streak, Epiblast, Anteroposterior axis, Gastrula, BrdU, DiI, Lagomorph

INTRODUCTION

The common element in different forms of vertebrate gastrulation is the generation of the germ layers. They establish (1) the internal milieu of the embryo in the form of the mesoderm, endoderm and neuroectoderm, and (2) the external cover in the shape of the epidermal ectoderm. Among these preliminary tissues the mesoderm is the earliest to be distinguished morphologically by epithelial-mesenchymal transition in the epiblast epithelium, a mechanism well conserved throughout the animal kingdom (Hay, 1995; Wodarz et al., 1995). In amniotes, the epithelial-mesenchymal transition and the continuous supply of new cells for the mesoderm, are both organised in the primitive streak, a band of increased cell density in the posterior half of the embryonic disc (Bellairs, 1986). As this band is longitudinally oriented in a round primitive embryonic 'body', its first appearance is well known as an early marker for the major (anteroposterior, craniocaudal) body axis, as well as for the start of gastrulation in general.

Interestingly, despite the conserved molecular moieties involved in vertebrate gastrulation (Kimelman and Griffin, 1998; Munoz-Sanjuan and Hemati-Brivanlou, 2001) and despite the common cellular mechanisms of mesoderm formation (Hay, 1995; Wodarz et al., 1995), the possibility exists that birds and mammals differ in the orchestration of mesoderm formation and gastrulation initiation, not least because their morphology is principally different from one another at the stage immediately preceding primitive streak formation: at this stage, the chick embryo is large (measuring about 4 mm in diameter and consisting of some 100,000 cells) and has a pronounced cellular density near the posterior margin (Koller's sickle) (Bachvarova et al., 1998). At the equivalent stage, the mammalian embryo measures – depending on the species – between 100 and 500 μm in diameter, consists of

between 900 and 5000 cells (Viebahn, 1999) and has a cellular density at the anterior pole, in form of the anterior marginal crescent (AMC). Posteriorly, the mammalian embryo is distinctly attenuated at pre-gastrulation stages (Viebahn et al., 1995). Biochemically, there seem to be differences, too, such as fibroblast growth factors (FGFs) being expressed and active at the posterior pole of the embryo in the chick (Storey et al., 1998; Streit et al., 2000) and *Xenopus* (Christen and Slack, 1997; Lamb and Harland, 1995) but anteriorly in the mouse (Rosenquist and Martin, 1995; Sun et al., 1999; Trumpp et al., 1999).

Gastrulation mechanisms have been studied in mammals mainly in the modern standard mammalian system for embryonic development: the mouse (Beddington and Robertson, 1998; Tam and Steiner, 1999). Unfortunately, a morphological landmark prior to the formation of mesoderm, i.e. at pre-gastrulation stages, is lacking in the living mouse embryo, as, owing to the shape of the 'egg cylinder', the AVE (equivalent to the AMC) is visible only using histological (Bonnevie, 1950; Kaufman, 1992) or biochemical (Rosenquist and Martin, 1995; Beddington and Robertson, 1998) methods in fixed embryos. Therefore, the presumptive area of mesoderm and primitive streak formation cannot be determined easily in the living mouse embryo prior to experimentation. The rabbit, however, as a lagomorph and similar to most mammals, has a flat embryonic disc. As in many other late-implanting species, the AMC can be seen as an anterior differentiation in the living embryo several hours before the primitive streak is formed (Viebahn et al., 1995) and may serve as landmark for investigation of the presumptive area of primitive streak (and mesoderm) formation.

In the face of the conflicting evidence on gastrulation between mammals and birds, we decided to examine several physiological characteristics of the rabbit embryo at around the stages of mesoderm formation to begin determining mechanisms of gastrulation initiation in a mammal. By RT-PCR we cloned a cDNA encoding 212 amino acids of a rabbit *Brachyury* homologue as an early specific marker of mesoderm formation (Papaioannou and Silver, 1998) in whole mount in situ hybridisation analysis. Cellular movements and proliferation in the presumptive area of primitive streak formation were studied in vitro using the vital dye DiI and the thymidine nucleotide analogue BrdU, respectively. We show that *Brachyury* expression is a sign of mesoderm progenitors scattered in a broad posterior extension of the epiblast several hours prior to the first visible signs of epithelial-mesenchymal transition and primitive streak formation. In this posterior area, which we propose to call PGE (posterior gastrula extension), the first primitive streak cells do not accumulate by local proliferation, but they migrate to this position from anterior parts of the embryo which show vigorous proliferative activity. We also note that the primordium of the primitive node is defined by *Brachyury* expression before the anterior parts of the primitive streak adjacent to the node acquire this capacity.

MATERIALS AND METHODS

Embryos

Uteri of New Zealand White rabbits were removed following the intravenous administration of an overdose (90 mg) of Narcoren® (Bayer Veterinärmedizin, München, Germany). Young embryonic discs (between 6.0 and 6.5 days post conception, d.p.c.) were dissected from blastocysts that were flushed from the uterus using warm (37°C) phosphate-buffered saline (PBS). Older embryonic discs (between 7.0 and 8.0 d.p.c.) were dissected from uteri immersed in PBS using iridectomy scissors. As gastrulating rabbit embryos look similar to chick embryos during primitive streak stages and beyond, the rabbit embryos removed at 6.5 to 8.0 d.p.c. were staged with reference to the system developed for the chick (Hamburger and Hamilton, 1993). For younger embryos (6.0 and 6.2 d.p.c.) additional stages (stages 1 and 2) were defined on the basis of morphology (Viebahn et al., 1995) and evidence obtained during the course of this study (Fig. 2).

Cloning of a rabbit *Brachyury* cDNA fragment

Messenger RNA from rabbit embryos at 7.0 d.p.c. was isolated using a commercial kit (Eurobio) and amplified using the SMART PCR cDNA synthesis kit (Clontech) and the following degenerated PCR primers, based on the mouse *Brachyury* sequence (Herrmann et al., 1990): 5′-CTCACCAACAAGCTCAATGGA (forward) and 5′-GATGGTACCATTGCTCACAGACC (reverse). Using 28 cycles at 95°C for 30 seconds, 55°C for 1 minute and 60°C for 2.5 minutes, a 635 bp fragment was cloned and a corresponding peptide sequence was derived using 'pileup' and 'lineup' (GCG program package version 10). Evolutionary analysis was performed with 'pileup', 'distances' (Jukes-Cantor distance) and 'growtree' with qualitatively similar results.

Whole-mount in situ hybridisation

Chemicals were purchased from Sigma (München, Germany) unless stated otherwise. Digoxigenin-labelled sense and antisense RNA was synthesised following standard protocols (Roche). In situ hybridisation protocols of Lowe et al. (Lowe et al., 1996) and Belo et al. (Belo et al., 1997) were adapted to the requirements of rabbit embryos: blastocysts were paraformaldehyde-fixed for 1 hour; embryonic discs were dissected in PBT, dehydrated and stored in methanol at –20°C up to 5 years. Protease treatment at RT was 5 minutes for 6.0 to 6.5 d.p.c. embryos and 10 minutes for older embryos. Hybridisation buffer contained 50% formamide, 1.3× SSC pH 4.5, 5 mM EDTA, 50 μg/ml tRNA, 0.2% Tween 20, 0,5% CHAPS and 50 μl/ml heparin. For prehybridisation (at 70°C for 1 hour) and hybridisation (70°C overnight) nylon baskets containing the embryos were transferred to sterile screw-top PVC tubes (Bibby-Sterilin, Staffordshire, UK). Following hybridisation with 0.4 μg/ml digoxigenin-labelled RNA probe (denatured for 2 minutes at 95°C) tissues were washed twice for 30 minutes in hybridisation buffer at 70°C and transferred to a prewarmed (70°C) 1:1 dilution of hybridisation buffer and MABT (100 mM maleic acid, 150 mM NaCl, 0.1% Tween 20, pH 7.5). Hybridised probes were visualised with anti-digoxigenin antibody coupled to alkaline phosphatase and BM-purple substrate (both Roche). The colour reaction was initiated by transferring embryos singly to organ culture dishes filled with the substrate and was allowed to proceed for 24 to 48 hours at room temperature. Tissues were photographed under a cover glass and stored in 100% glycerol at 4°C.

DiI labelling

Blastocysts with intact zona pellucida (6.2 d.p.c.) were submerged in HAM's F10 medium containing 20% FCS and manoeuvred, with the embryonic disc lying near the top, onto a stainless steel washer with an inner diameter of 2 mm. Medium was aspirated to the point that the embryonic disc area of the blastocyst was exposed to air. The zona pellucida, which is still robust at this stage and prevented the blastocyst from collapsing during the holding and the ensuing injection procedure, was penetrated over the injection site with a micropipette (2 μm inner diameter at the tip) held at ~45° to the surface of the embryonic disc (Fig. 1A). In some cases, a piezoelectric drill

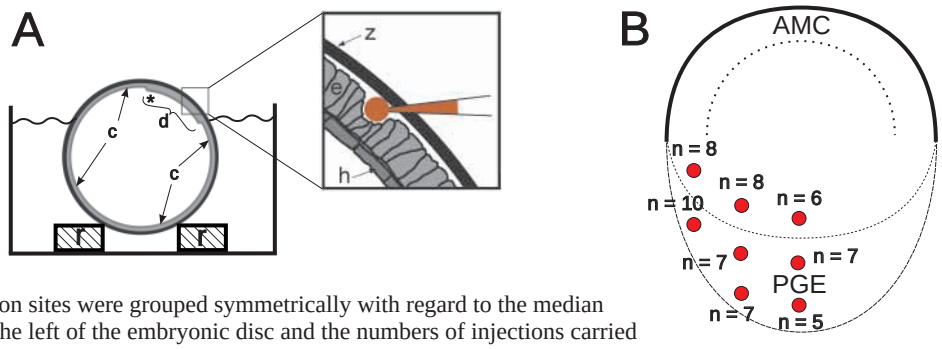

Fig. 1. Experimental set-up for DiI injection shown in transection. (A) Whole 6.0 d.p.c. blastocyst (c) with intact zona pellucida (z) held on a metal ring (r). Embryonic disc (d) with its anterior margin (*) projects above the fluid level. The tip of the injection pipette and the injected dye lie in the space between epiblast (e) and zona pellucida (z), thereby avoiding contact with the hypoblast (h). (B) DiI injection sites just anterior to and within the PGE. Lateral injection sites were grouped symmetrically with regard to the median plane; for simplicity, they are shown only on the left of the embryonic disc and the numbers of injections carried out in corresponding injection sites are accumulated.

(Burleigh, NY, USA) was used to achieve penetration of the zona and to avoid penetration of the cellular layers (epiblast and hypoblast) of the embryonic disc. Using a microsyringe, between 0.5 and 2 pl of the lipophilic carbocyanine dye DiI (1,1′-dioctadecyl-3,3,3′-tetramethyl indocarbocyanine perchlorate, Molecular Probes) dissolved in corn oil (Terasaki and Jaffe, 1993) was injected into various parts of the PGE (Fig. 1B) to label migrating epiblast cells. In each blastocyst, additional DiI deposits were placed anteriorly or in extra-embryonic positions to serve as reference points for the orientation of embryonic disc after culture. Injected blastocysts were transferred to fresh medium for incubation at 37°C under 5% CO_2 for 8 (n=4 blastocysts), 12 (n=17) or 16 (n=10) hours. At the beginning and at the end of the culture period, embryonic discs were photographed under dark-field optics to verify the position of the DiI-oil deposits. After culture, blastocysts were fixed in 4% PFA in PBT for 1 hour. The zona pellucida was removed with flame-polished tungsten needles and care was taken not to remove the DiI deposits, which were not held in place after removal of the zona but, in most cases, adhered sufficiently to the epiblast to remain in situ until mounting of the specimen for microscopy. To this end, the embryonic discs were dissected in PBT, transferred to a glycerol drop on a microscope slide, flattened with tungsten needles and photographed under a cover glass in an upright microscope (Axioplan, Zeiss, Jena, Germany).

After obtaining initial results on the amount of dye spread (100 to 200 μm), some embryos were injected with multiple dye deposits that were placed well apart. Of 41 injected embryos, 34 had developed normally to early or late primitive streak stages depending on the length of the culture period chosen. Thirty-one embryos were included in the present study (58 injections). To verify the position of labelled cells within cell layers (i.e. epiblast/ectoderm, hypoblast and mesoderm), some embryonic discs were incubated – after culture and fixation – for 10 minutes in the fluorescent DNA stain 4′-6′diamidino-2-phenylindol (DAPI; SERVA, Heidelberg, Germany) at 0.1 μl/ml in PBS, photographed, embedded in OCT freezing medium (Tissue Tec, Naperville, USA) and cut at –20°C (10 μm) in a cryostat. Alternatively, DiI labelling was photoconverted into 3,3′-diaminobenzidine precipitates exactly as described by Stern (Stern, 1990) using 500 μg/ml DAB (DAB-tablets, Sigma) in a microchamber on a microscope slide made of four coverglass chips serving as lateral strutts and a 20×20 mm cover glass serving as a lid. Maximum fluorescence exposure was applied using a 40× oil Neofluar lense (Zeiss, Jena, Germany) at 488 nm for at least 30 minutes or until the DiI labelling had completely faded and general background staining started to cover up specific labelling. Evaporation of the incubation solution during the photoconversion was avoided by keeping the edges of the microchamber covered with DAB-Tris solution. Photoconverted embryonic discs were dehydrated using methanol series, embedded in Technovit® and cut at 5 μm.

BrdU labelling

Freshly isolated whole blastocysts were incubated in HAM's F10 medium containing 20% FCS at 37°C under 5% CO_2 for 30 minutes, transferred to 1 ml prewarmed HAM's F10 medium containing 20% FCS and 0.1 mM BrdU (Sigma) for a further incubation of 10 minutes. BrdU incorporation was stopped by adding 1 ml 1% glutaraldehyde in PBS. The embryonic discs were dissected in PBT and washed three times for 10 minutes in PBT followed by incubation for 60 minutes in 0.05% trypsin in 0.1% $CaCl_2$, pH 7.8. After a further three 10 minute washes in PBT, tissues were placed in 2 N HCl for 30 minutes, washed three times in PBT, incubated in 1% H_2O_2 for 30 minutes, washed three times in PBT and then incubated in the primary antibody (Bu 20a, DAKO, Hamburg) diluted to 1:250 in PBT for 2 hours at room temperature or overnight at 4°C. After three 10 minute washes in PBT, tissues were incubated at room temperature for 60 minutes in the secondary antibody (GAM/Ig/PO, Nordic, Düsseldorf, Germany) at 1:100 in PBT, washed three times in PBT and incubated for 10 minutes in DAB substrate. Labelled embryonic discs were photographed as whole-mounts in glycerol before they were fixed and embedded for histological analysis. For controls, blastocysts were incubated in HAM's F10 containing 20% FCS without addition of BrdU; alternatively, blastocysts that had been incubated in the presence of BrdU were immunohistochemically stained omitting the primary antibody. All controls gave negative results.

Morphometry

Using an eyepiece graticule (number 434008-9901, Zeiss, Jena, Germany) and the 25× lens in an Axiomat upright microscope (Zeiss) the labelling index (number of labelled nuclei divided by the number of unlabelled nuclei) was calculated in selected boxes of the graticule in three late stage 2 embryos prior to Araldite® embedding (for further details, see Fig. 8 and Results). P-values were calculated as Bonferroni-adjusted pairwise comparisons in a random effect ANOVA model (Lindman, 1992).

Histology

Staging and positions of labelling reactions were histologically analysed on semi-thin plastic sections: after in situ hybridisation, tissues were dehydrated in methanol, embedded at 4°C in Technovit® 8000 (Heraeus Kulzer, Werheim, Germany) in empty tablet moulds and sectioned at 5 μm using glass knives. BrdU-labelled tissues were fixed in 1% glutaraldehyde and osmium tetroxide, embedded in Araldite® and sectioned at 1 μm (Viebahn et al., 1995).

RESULTS

Gastrulation in the rabbit embryo

Prior to primitive streak formation, two hitherto undescribed stages can be distinguished in the living rabbit embryo under the dissecting microscope (Fig. 2): stage 1 embryonic discs are characterised by a smooth, sharp contour anteriorly, which

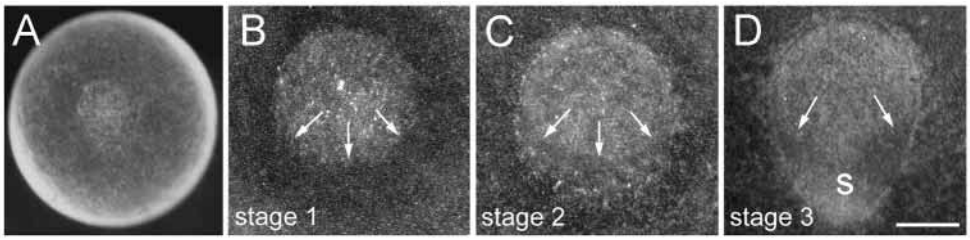

Fig. 2. Early gastrulation stages of the rabbit embryo. (A) Whole blastocyst at a pre-primitive streak stage (6.0 d.p.c.); the plane of focus is on the blastocyst wall containing the embryonic disc. (B) Stage 1 with smooth anterior contour (epiblast-trophoblast junction) of the disc indicating the position of the AMC. The posterior contour (arrows) is more ragged. (C) Stage 2 with the 'posterior gastrula extension' (PGE), a sickle-shaped area of reduced density that lies posteriorly to the ragged posterior contour (arrows) seen at stage 1. (D) Stage 3 with primitive streak (s) in the midline of the PGE. The former posterior margin is still visible (arrows). Scale bars: 1.3 mm in A; 250 μm in B,C; 270 μm in D.

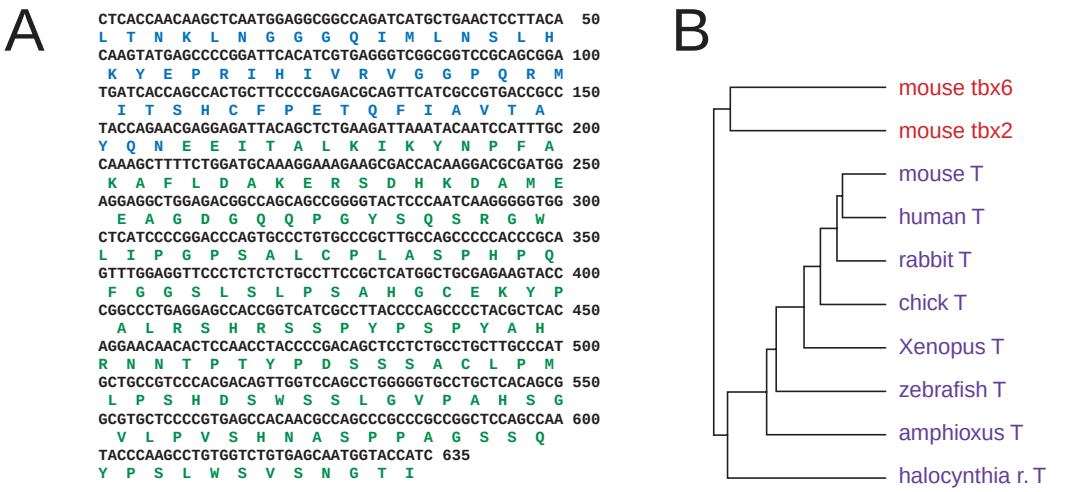

Fig. 3. Rabbit *Brachyury* cDNA fragment and predicted protein sequence (A), interspecific comparison of protein sequence (B) and cladogram (C) show that rabbit cDNA and protein sequence fall within the *Brachyury* (*T*)-family. Amino acids in the T-box are shown in blue, other residues in green. Deviations from the consensus sequence are highlighted in red. Sequences were taken from the following papers: mouse (Herrmann et al., 1990); human (Edwards et al., 1996); chick (Kispert et al., 1995); *Xenopus* (Smith et al., 1991); zebrafish (Schulte-Merker et al., 1992); *Amphioxus* (Holland et al., 1995); *Halocynthia roretzi* (Yasuo and Satoh, 1998); *Hydra* (Technau and Bode, 1999); mouse *tbx2* (Bollag et al., 1994); mouse *tbx6* (Agulnik et al., 1996).

marks the position of the anterior marginal crescent (AMC) (Viebahn et al., 1995); in comparison, the posterior contour of the embryonic disc is more ragged and indistinct (Fig. 2B). Stage 2 has a sickle-shaped elongation at the posterior pole, which does not show a density typical of the primitive streak yet but appears, on the contrary, less dense than the remaining anterior part of the embryonic disc. The ragged posterior margin of stage 1 now marks a border within the embryonic disc, namely between the anterior dense area and the attenuated posterior extension (Fig. 2C). Because the experimental analysis of this study shows that this posterior area contains the presumptive mesoderm cells and may have an important functional role in the early gastrulation process, this area is addressed as 'posterior gastrula extension' (PGE) in the present report. Stage 3 begins with the appearance of a median density within the PGE, this density being a reflection of the first mesoderm cells appearing in the compartment between the epiblast and the hypoblast (Fig. 2D).

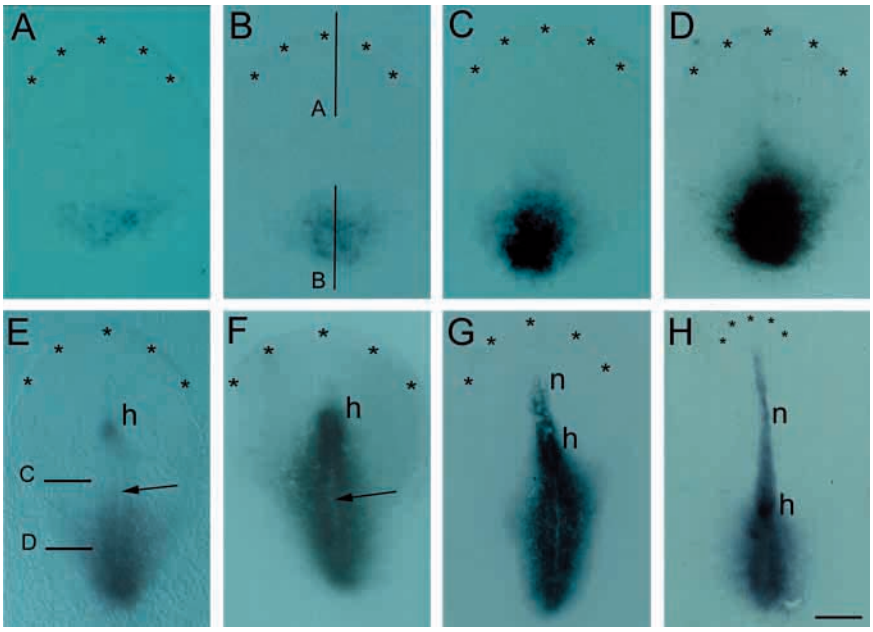

Fig. 4. *Brachyury* expression during gastrulation (between 6.0 and 7.5 d.p.c.) as determined by in situ hybridisation; position of the AMC is indicated by asterisks. Bars and letters indicate position of sagittal sections shown in Fig. 5. (A,B) Stage 2, *Brachyury*-expressing cells lie singly at first (A) and a little later in a coherent patch (B) in the PGE. (C,D) Strong *Brachyury* expression in the emerging (C) and definitive (D) primitive streak at stage 3. (E) Early stage 4 with lack of *Brachyury* expression in the anterior half of the primitive streak (anterior to arrow) but distinct expression in the primordium of Hensen's node (h). (F) Late stage 4 with node fully formed and strong *Brachyury* expression along the entire primitive streak. Arrow as in E. (G,H) Stage 5 and 6 with *Brachyury* expression also in notochordal process (n). Scale bars: 130 μm in A-D; 160 μm in E-G; 290 μm in H.

Structure and expression of rabbit *Brachyury*

The rabbit *Brachyury* orthologue obtained by RT-PCR is 635 bp long (Accession Number, AF102131). The predicted amino acid sequence begins at position 148 of the consensus Brachyury protein (approximately halfway through the T-box) encodes 212 amino acids and shows 95% homology with the mouse and human sequences (Fig. 3). The clone belongs to the *T*-family and is most closely related to mouse and human *Brachyury* (Fig. 3B).

Transcripts of the *Brachyury* fragment are first detected in stage 2 embryos, when the first signs of the PGE become apparent (cf. Fig. 2C). In the PGE of early stage 2 embryos, a few single *Brychyury*-positive cells lie among weakly positive and negative cells, creating a salt-and-pepper-like pattern (Fig. 4A). In late stage 2 embryos, positive cells lie in small, almost confluent groups of cells that cover a larger area along the anteroposterior axis when compared with early stage 2 embryos (Fig. 4B). Serial sagittal sections confirm the posterior position of these labelled cells by the presence of the increased cellular height and density in epiblast and hypoblast (typical of the AMC) at the margin opposite from the *Brachyury* expressing epiblast cells (cf. Fig. 5A,B). Hypoblast cells are not labelled and, importantly, mesoderm cells that typically lie in the space between the epiblast and the hypoblast at the posterior margin at stage 3 cannot be detected. The intensity of the hybridisation reaction in the epiblast cells varies in accordance with the variable, at early stages salt-and-pepper-like, pattern seen in the whole mount views (Fig. 4A).

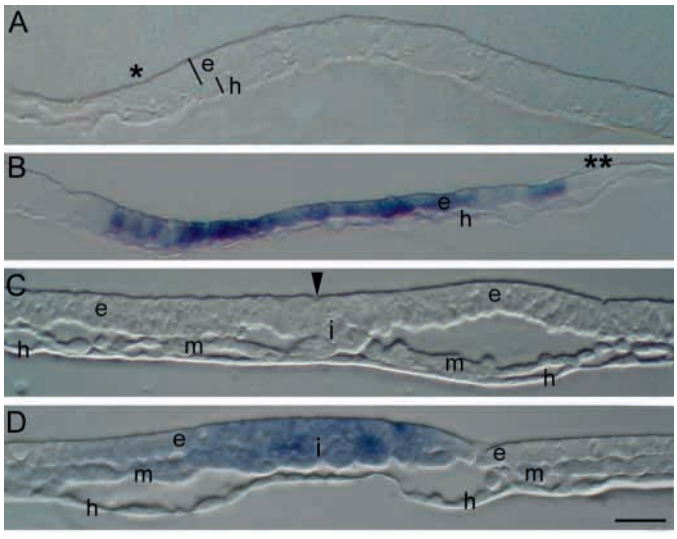

Fig. 5. Cellular distribution of *Brachyury* expression as seen in sagittal (A,B) and horizontal (C,D) semi-thin sections from specimens in Fig. 4. (A,B) At stage 2, *Brachyury* transcripts are confined to epiblast cells (e) in the posterior third of the embryonic disc. Anterior (A) and posterior (B) margins (epiblast-trophoblast junction) are marked with single and double asterisks, respectively. (C,D) No *Brachyury* expression in epiblast or mesoderm of the anterior half of the primitive streak at stage 4 (arrowhead marks primitive groove in C) but strong *Brachyury* expression in the posterior half of the primitive streak (D) in epiblast (e) and ingressing mesoderm (i). Scale bar: 40 μm.

As soon as mesoderm cells can be recognised in median sagittal sections, i.e. at stage 3, *Brachyury*-expressing cells are confluent and cover a large part of the PGE (Fig. 4C). As the primitive streak lengthens during further development, the area of the *Brachyury* expression spreads anteriorly about half-way along the primitive streak (Fig. 4D). Intriguingly, as the primordium of Hensen's node appears at stage 4, *Brachyury* expression is detected in the node area, while it is still absent in the anterior half of the primitive streak (Fig. 4E). That the epithelial-mesenchymal transition typical of the streak is continuously present up to the node area at this stage, is seen in horizontal sections through the anterior half of the primitive streak (Fig. 5C). Within the posterior half of the streak, both mesoderm and epiblast are *Brachyury*-positive (Fig. 5D). Mesoderm cells lose *Brachyury* expression as they leave the area of the primitive streak to more lateral positions (Fig. 5D). Similarly, both mesoderm and epiblast of the node express *Brachyury* (not shown). At the late stage 4, the areas of *Brachyury* hybridisation reaction within the primitive streak and node are continuous (Fig. 4F). At stage 5, when the pre-chordal mesoderm and head process can be distinguished and at stage 6 (early head fold stage), *Brachyury* expression extends to the anterior tip of the head process (Fig. 4G,H).

Vital dye labelling

After 8 hours in culture, embryonic discs develop from stage 2 to early stage 3 (compare Fig. 2C,D with Fig. 6A,B). Similarly, after 12 and 16 hours in culture, embryonic discs develop to mid and late stage 3, respectively (Fig. 6E,I). During the culture, the deposits of injected DiI generally move slightly

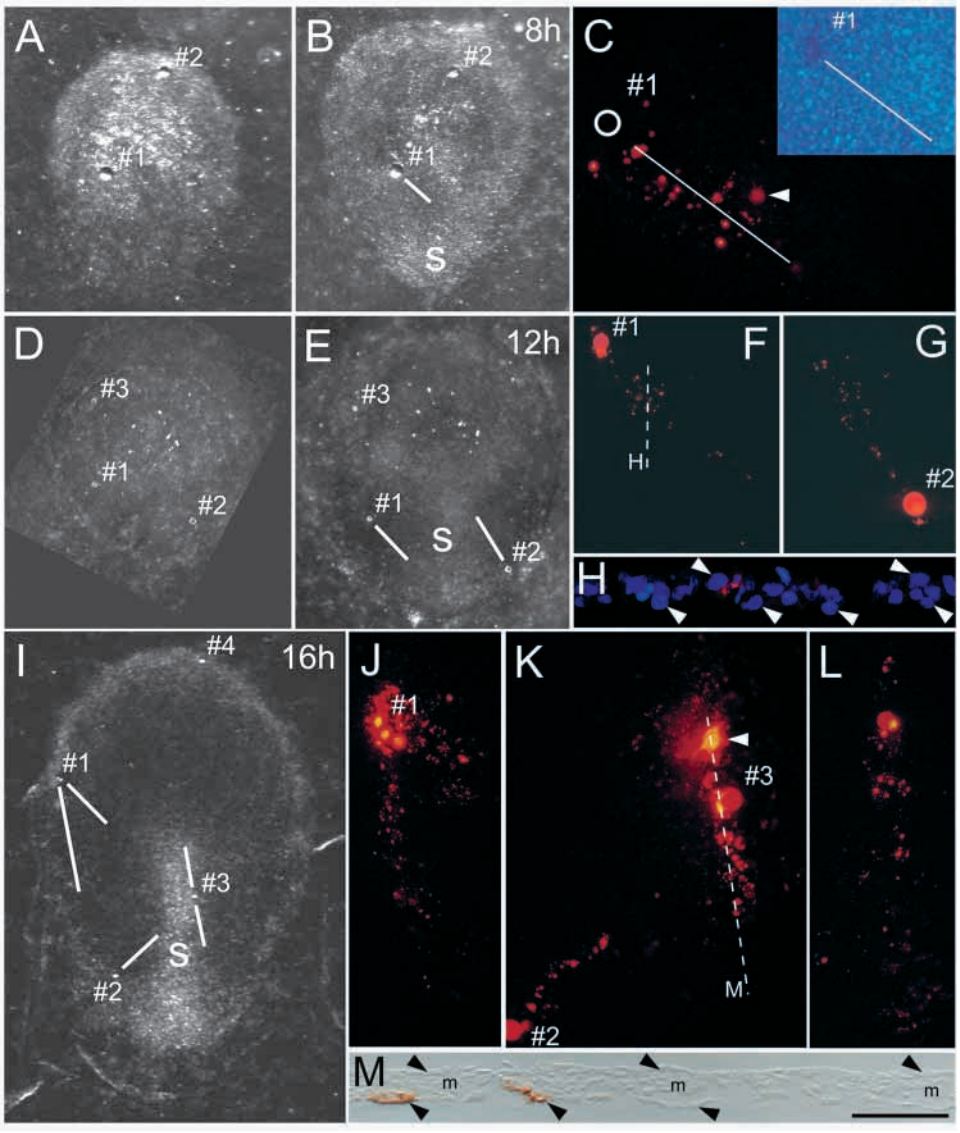

Fig. 6. Cellular migration in the posterior half of 6.2 d.p.c. embryonic discs as determined by DiI labelling and culturing for 8 (A-C), 12 (D-H) and 16 hours (I-M). Dark-field photographs taken immediately after the injection (A,D) and after culture (B,E,I). DiI deposits are numbered in the order of injection in each embryo and appear with a shadow image under asymmetrical dark field illumination (A,B,I) and ring-like under circular illumination (D,E). Direction and distance of dye spread (white bars in B,E,I), as determined in fluorescence micrographs of embryonic discs mounted on microscope slides (C,F,G,J-L). (C) Dye spread in posteromedial direction (white line) following injection of bolus #1 (position indicated by white ring) placed mediolateral and anterior to the PGE of embryo shown in A,B. The DiI bolus was lost during mounting after removing the zona pellucida but left a mark of reduced cellular density seen in DAPI labelling (see inset). Translocated DiI appears as minute spots in the area of the PGE; these lie in one plane except for some spots that produce typically larger out-of-focus images (arrowhead). (F,G) Dye spread in posteromedial (F) and anteromedial direction (G) after injection anteromedially (#1) or posteromedially (#2) in the PGE in the embryo shown in D,E. (H) DAPI-labelled frozen section (position marked with broken line in F) showing epiblast (arrowheads pointing downwards) and not hypoblast cells (arrowheads pointing upwards) being labelled with DiI. (J,K) Dye spread after injections near the lateral margin anterior to the PGE (#1), posteromedially in the PGE (#2) and in the anterior centre of the PGE (#3) in the embryo shown in I. Injection #3 was inadvertently positioned between epiblast and hypoblast and gave rise – in addition to the typical spot-like labelling (compare C,F,G) – to complete cells being labelled (e.g. arrowhead). (M) Nomarski image of semi-thin section (position marked with broken line in K) of the specimen in K after photoconversion. DAB precipitates are found in the strongly labelled (hypoblast) cells only (arrowheads as in H; m, mesoderm). (L) Posterior and some anterior dye spread after injection similar to #3 in I and after development to late stage 3 (dark-field view of embryo not shown). Scale bars: 300 μm in A,B,D,E,I; 55 μm in C (inset 80 μm); 75 μm in F,G; 20 μm in H; 70 μm in J-L; 35 μm in M.

apart and to more posterior positions, thereby keeping their relative position to each other (compare Fig. 6A,B with Fig. 6D,E). The amount of dye spread as a result of labelled migratory cells varies according to the length of the culture period: the distance of maximal linear translocation of the dye (white bars in Fig. 6) and its lateral spread are about 120 μm and 40 μm, respectively, after 8 hours in culture (Fig. 6C), and 200 and 80 μm, respectively, after 16 hours (Fig. 6J). Translocated dye appears as minute spots of fluorescence that rarely can be allocated to individual cells in whole-mount preparations. However, frozen sections prove such DiI spots to belong to the epiblast layer (Fig. 6H). Only occasionally are whole cells outlined by the dye (Fig. 6K). These are invariably found to belong to the hypoblast and are the result of DiI injections inadvertantly placed in the space between epiblast and hypoblast. However, this strong DiI labelling of whole cells gives rise to DAB precipitates after photoconversion (Fig. 6M), while photoconversion of the spot-like fluorescence seen in neighbouring cells (Fig. 6K) does not produce DAB precipitates above background level (Fig. 6M) even after maximal exposure to the light source of the fluorescence microscope (data not shown).

Following injection into lateral areas anterior to the anterior border of the PGE (Fig. 1B) most DiI spots are found in more posteromedial positions, i.e. they have crossed the anterior border of the PGE (Fig. 6C,J). Injections placed most laterally and anterior to the PGE, in addition, lead to pronounced translocation of the dye to more medial positions anterior to the PGE (Fig. 6J). Lateral injections just posterior to the anterior border of the PGE result in posteromedial translocation of the dye within the PGE (Fig. 6F). Injection in the posterolateral part of the PGE, however, results in DiI translocation to anteromedial positions (Fig. 6G and #2 in Fig. 6K). Deposition of DiI in the median plane, i.e. in the presumptive area of the primitive streak, results in anterior and posterior translocation of the dye near the median plane when injections hit the anterior part of the PGE (#3 in Fig. 6K, Fig. 6L). Median injections further anteriorly or posteriorly result in predominantly anterior and posterior translocations, respectively (data not shown).

Proliferation analysis

In surface views of BrdU-labelled blastocysts the embryonic discs stand out at all stages as heavily labelled against the largely unlabelled extra-embryonic tissue (Fig. 7A-E) and labelled nuclei can be easily distinguished from unlabelled ones at a high magnification (Fig. 7A′,A″). Specifically, stage 1 embryos (*n*=3) show an even distribution of labelled nuclei throughout the embryonic disc (Fig. 7A). In early stage 2 embryos (*n*=7), an increase in labelling intensity appears in a transverse band at the level of the middle third of the embryonic disc, while the area of the PGE shows about the same labelling intensity as the anterior third of the embryonic disc (Fig. 7B). In late stage 2 embryos (*n*=9), the PGE has a similar labelling intensity as at the previous stage, while the remainder of the embryonic disc now shows a ring of increased labelling that covers a narrow band at the anterior margin of the disc and is continued in a wider band anterior to the border to the PGE (Fig. 7C). Inside this ring, labelling appears about as intense as in the PGE. Absence of mesoderm cells in these late stage 2 embryos is confirmed in serial sagittal sections (Fig. 7F). At the onset of mesoderm formation, as seen by the presence of mesoderm cells in sagittal sections (Fig. 7G) [i.e. at early stage 3 (*n*=4)], BrdU-labelling is more intense within the ring-like structure seen at the late stage 2. It is also strong in the emerging primitive streak (Fig. 7D). With the streak elongating in late stage 3 embryos (*n*=4), labelling remains intense in the primitive streak and in the ring-like structure of the anterior part of the embryonic disc. This ring of strong labelling appears to be discontinuous at the level of the anterior

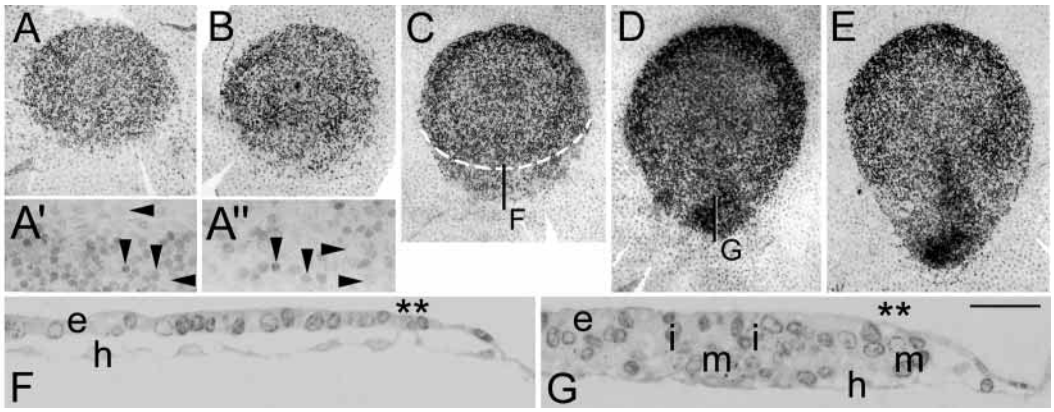

Fig. 7. Cellular proliferation after pulse BrdU labelling in vitro. (A) Stage 1 with uniform labelling of the embryonic disc and background labelling in the bordering extra-embryonic tissues. A′ and A″ anterior and posterior border, respectively, of embryo in A to show unlabelled (horizontal arrowheads) and labelled (vertical arrowheads) nuclei. (B,C) Early and late stage 2, respectively, with PGE (anterior border marked with broken line) less densely labelled than remainder of embryonic disc. Vertical bar indicates position of section shown in F. (D,E) Early and late stage 3, respectively, with intense labelling of AMC (compare with C) and primitive streak. Vertical bar indicates position of section shown in G. (F,G) Posterior sagittal sections from embryo in C,D, respectively, confirm the absence of mesoderm cells at stage 2 (C,F) and vigorous mesoderm ingression at early stage 3 embryo (D,G). At stage 2 (F), labelled nuclei are almost exclusively found in the epiblast (e) and at stage 3 (G), the hypoblast (h) is still unlabelled, while many nuclei are labelled in epiblast (e), in ingressing (i) and definite (m) mesoderm. Double asterisks mark posterior embryonic border at the junction with the extra-embryonic trophoblast. Scale bars: 250 μm in A-E; 60 μm in A′ and A″; 30 μm in F,G.

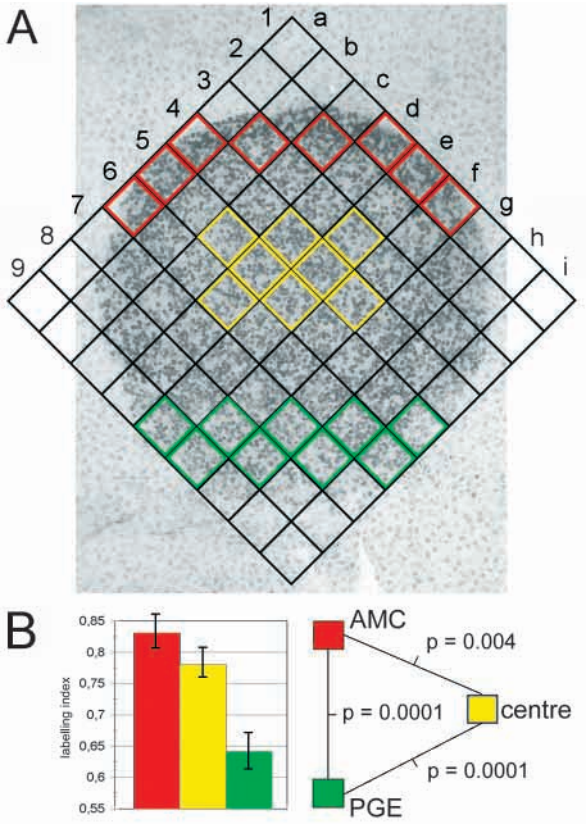

Fig. 8. Morphometric analysis of BrdU labelling at stage 2.
(A) Position of grid boxes used for counting in anterior (red), central (yellow) and posterior (green) regions of the embryonic discs.
(B) Graphical comparison and confidence values between average labelling indices obtained in these three regions.

extremity of the primitive streak (Fig. 7E). In addition, an area that lies lateral to the elongating primitive streak and is equivalent to the PGE of earlier stages (Fig. 2D) is less heavily labelled than the rest of the embryonic disc (Fig. 7E). Sagittal sections show that the labelled nuclei are almost exclusively found in the epiblast at stage 2 (Fig. 7F) and in epiblast and mesoderm at stage 3 (Fig. 7G). Only few hypoblast cells are labelled in any of the specimens.

Morphometric analysis

Labelling indices determined in three representative areas of the embryonic disc at stage 2 (Fig. 8A) show that the average value in the PGE (0.64) is significantly lower (P=0.0001, Fig. 8B) than the ones obtained in the AMC (0.83) or in the centre of the embryonic disc (0.78). The difference between the centre and the anterior margin is also significant, albeit with a lower confidence value (P=0.004).

DISCUSSION

Early stages of mammalian gastrulation

This report identifies a new morphologically and physiologically distinct posterior area of the epiblast that seems to play an important role during the initiation of gastrulation. The term ‘posterior gastrula extension’ was coined and used to define the second of two new stages leading up to primitive streak formation (Fig. 2). Because an area with similar morphological characteristics as the PGE may also be seen in suitable lateral views from prestreak mouse embryos (Downs and Davies, 1993) and because evidence for the presence of the AMC is available in a number of mammalian species (Viebahn, 1999) it may be assumed that the two stages described here are representative for early mammalian gastrulation.

Mesoderm specification by *Brachyury* expression

The *Brachyury* fragment cloned here is considered a reliable marker for characterising mesoderm progenitors in the rabbit, as sequence and expression pattern are well conserved. The early *Brachyury* expression in the epiblast fits the expression pattern in pre-streak stages of mouse (D. Stott, personal communication) and chick (Kispert and Herrmann, 1994; Knezevic et al., 1997). Taking into account the differences in gastrula shape between the rabbit (flat disc) and the mouse (cup-shaped egg cylinder), the spread-out *Brachyury* expression pattern in the rabbit seems to be equivalent to the circular pattern described in a whole-mount view for the mouse (Thomas and Beddington, 1996). The mosaic pattern seen at the very beginning of *Brachyury* expression is reminiscent of the HNK1-pattern heralding mesoderm formation in the chick epiblast (Stern and Canning, 1990) and suggests that intraepithelial cell movements are needed to assemble mesoderm progenitors in the forming primitive streak in mammals as well.

The transient division of the primitive streak by *Brachyury* expression in the posterior but not the anterior half at the early node stage is also seen in the bovine embryo (Hue et al., 2001) and, to some degree, in the chick (Knezevic et al., 1997). Several genes also show graded expression along the anteroposterior axis of the primitive streak, such as *Evx1* (Dush and Martin, 1992), *MesP1* (Saga et al., 1996), *LCR-F1* (Farmer et al., 1997), *mrg1* (Dunwoodie et al., 1998), *caudal* (Epstein et al., 1997) and *plp2* (Milde et al., 2001). In the case of *Brachyury*, this pattern is directly matched by anteroposterior differences in the regulatory control of the gene (Clements et al., 1996), possibly through the action of FGFs (Griffin et al., 1995). In addition, epiblast cell fates are different along the primitive streak (Garcia-Martinez et al., 1993; Lawson et al., 1991) and organiser potential varies from anterior to posterior [the early gastrulation organiser EGO (Tam and Steiner, 1999); trunk and tail organisers (Griffin et al., 1995; Knezevic et al., 1998)]. However, specific posterior development in the streak and, not least, the characteristics of the T mutation (Wilkinson et al., 1990) revive old ideas of a ‘posterior node’ playing an important role in the formation of extra-embryonic tissues and tail structures (Florian, 1933; Seidel, 1960).

Cellular migration preceding primitive streak formation

The predominant directions of DiI translocations observed during development from stage 2 to stage 3 in vitro are summarised schematically and ‘translated’ into cell movements at stage 2 in Fig. 9: each arrow represents the cumulated results obtained at each position chosen for injection (Fig. 1B). A technical point worth mentioning is that the application of DiI in an oil (rather than water-based)

suspension produces compact dye deposits that are convenient to localise and offer relatively small areas of contact to the cells to be labelled. Together with short contact times of fast moving cells such as epiblast, this can be assumed to lead to partial labelling of cell membranes and, hence, to the spot-like appearance of translocated dye. Only in combination with long contact times of cells moving slowly, such as hypoblast, is the more familiar sheet-like and complete labelling of cell membranes produced.

The suggested cell movements enlarge the anterior, round part of the stage 2 embryonic disc almost concentrically and continue to supply cells to the PGE, which was established beyond the posterior border of the embryonic disc between stage 1 and 2 (Fig. 2B,C). In addition, complex movements within the PGE start to shape the PGE in a characteristic fashion: epiblast cells within and lateral to the presumptive area of the primitive streak move either anteriorly or posteriorly (Fig. 9) and create two whirl-like centres with opposing 'circle currents' on both sides of the midline. These movements lead to the elongation of the PGE in both anterior and posterior directions and later, when the streak emerges and elongates, they contribute to the narrowing of the embryonic disc seen in its posterior half (Fig. 4D-F). In principle, these movements conform with those described in the pre-streak chick embryo as 'non-random cell mixing' (Hatada and Stern, 1994). They also support the finding that mesoderm progenitor cells lie dispersed (mosaic-like) at first, as seen in *Brachyury* expression (Fig. 4A) and HNK1 labelling (in the chick epiblast) (Stern and Canning, 1990) and then move past non-progenitor cells towards the midline to form the primitive streak.

The cellular migration pattern in the pre-streak mammalian embryo pre-empts the pattern seen during overt gastrulation: after primitive streak formation, cells continue to move centripetally and posteriorly towards the primitive streak [rabbit (Daniel and Olson, 1966); mouse (Lawson et al., 1991)]. Unfortunately, which fate these cells take on within the mesoderm (or the notochord) could not be observed in the present investigation because the culture period could not be extended much beyond early node stages because of technical problems (rupture of zona pellucida and disintegration of the embryonic disc shape) (C. V., C. S., S. A. M. and M. B., unpublished).

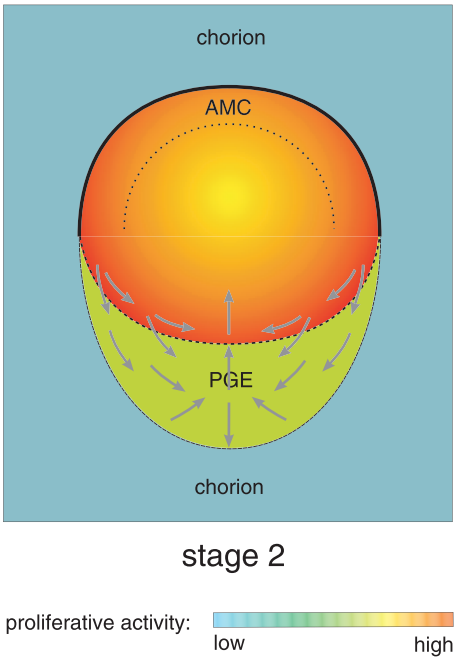

Fig. 9. Integration of the migratory, proliferative and differentiation behaviour of epiblast cells at the late pre-primitive streak stage (stage 2) of mammalian gastrulation: differentiation towards a mesodermal fate as revealed by *Brachyury* expression in the epiblast of the PGE (compare with Fig. 4A) occurs under the condition of a reduced proliferative activity (light green) in the PGE, when compared with high proliferative activity (yellow-red) in the remainder of the embryonic disc. This is accompanied by cellular rearrangement (grey arrows) between anterior and posterior areas of the embryonic disc and within the PGE to form the primitive streak. Each arrow represents the summary of results obtained for the individual sites chosen for injection anterior to and within the PGE (compare with Fig. 1B). Extra-embryonic tissues (chorion) are depicted as largely non-proliferative (light blue).

Proliferation and the start of gastrulation

The proliferative values obtained here appear exceptionally high with 64 to 83% of epiblast cells in S phase during any 10 minute period of early gastrulation. However, an identical labelling pulse of 10 minutes was used by Mac Auley et al. (Mac Auley et al., 1993) with H3-Thymidine for a stathmokinetic analysis of the cell cycle structure in the primitive streak of the rat. The values calculated from these experiments closely match our figures, in that S phase was found to use about 72% of a complete cell cycle (Mac Auley et al., 1993), which in highly proliferative areas was found to be as short as 3 hours. As most mammals grow rapidly during and preceding gastrulation, short cycling times might, indeed, be a necessity and the ventral buckling of the pre-gastrulation rodent embryo to form the egg cylinder may be regarded as the obvious result of this vigorous proliferative activity.

In the face of proliferation studies which are available for later (primitive streak) stages and in which centres of increased proliferation are found in the primitive streak once it is formed [mouse (Snow, 1977); rat (Mac Auley et al., 1993); chick (Stern, 1979; Sanders et al., 1993), our results can be directly compared only with results obtained at the pre-streak stages in the chick (Sanders et al., 1993; Zahavi et al., 1998). However, these results obtained in the chick are contradictory, possibly owing to the complex architecture in the posterior quadrant of the avian embryonic disc (Bachvarova et al., 1998). At least, the interspecific comparison confirms the reduced level of proliferation in the hypoblast (Mitrani, 1984) and it may be speculated that proliferation does not play a major role at the beginning gastrulation in the chick because cells are already present in sufficient numbers and need only to be redistributed through morphogenetic movements, which may be extensive (Stern, 1990).

With respect to initiating gastrulation by mesoderm differentiation, exciting parallels exist to *Drosophila*. Here, proliferation is reduced locally by the action of the *tribbles* gene (coding for a serine/threonine kinase) (Seher and Leptin, 2000; Großhans and Wieschaus, 2000; Mata et al., 2000) to enable the start of the mesoderm differentiation programme (as analysed by *twist* activity). Extrapolated to the present results

this would suggest that, in a mammal, epiblast cells have to leave the belt of proliferation anterior to the PGE to be able to respond to signals that initiate the *Brachyury*-driven mesoderm formation programme such as nodal (Zhou et al., 1993; Conlon et al., 1994) or Wnt3 (Liu et al., 1999). Intriguingly, *Brachyury* expression in the presumptive area of Hensen's node (Fig. 4E), too, lies in an area of reduced proliferative activity (Fig. 7E) so that the *Brachyury* programme in node cells may also be started by cell movement away from areas of high proliferation (Fig. 9). In the chick, however, distinct differences in proliferative activity do not seem to exist at pre-streak stages and in the absence of regulatory control by proliferation, alternative mechanisms such as growth factors and their inhibitors (e.g. chordin) (Streit et al., 1998) attain a more important role. In an evolutionary context, entering gastrulation using different mechanism may be the last ontogenetic afterplay of the differences seen in egg size and cleavage patterns, for example, between birds and mammals. Together with divergent development after the phylotypic stage, this phenomenon has been known since von Baer's time as the 'bottle neck' (Duboule, 1994) and it seems as if initiation of gastrulation already marks the beginning of the narrow part of this neck.

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