Differentiation 78 (2009) 301–311

Contents lists available at ScienceDirect

Differentiation

journal homepage: www.elsevier.com/locate/diff

Axial differentiation and early gastrulation stages of the pig embryo ☆

Romia Hassoun [a], Peter Schwartz [a], Kerstin Feistel [b], Martin Blum [b], Christoph Viebahn [a,*]

[a] Department of Anatomy and Embryology, Göttingen University, Kreuzbergring 36, 37075 Göttingen, Germany
[b] Institute of Zoology, Hohenheim University, Stuttgart, Germany

ARTICLE INFO

Article history:
Received 4 March 2009
Received in revised form
20 July 2009
Accepted 23 July 2009

Keywords:
Hypoblast/visceral endoderm
Epiblast
Mesoderm
Epithelial–mesenchymal transformation
Amnion
Trophoblast

ABSTRACT

Differentiation of the principal body axes in the early vertebrate embryo is based on a specific blueprint of gene expression and a series of transient axial structures such as Hensen's node and the notochord of the late gastrulation phase. Prior to gastrulation, the anterior visceral endoderm (AVE) of the mouse egg-cylinder or the anterior marginal crescent (AMC) of the rabbit embryonic disc marks the anterior pole of the embryo. For phylogenetic and functional reasons both these entities are addressed here as the mammalian anterior pregastrulation differentiation (APD). However, mouse and rabbit show distinct structural differences in APD and the molecular blueprint, making the search of general rules for axial differentiation in mammals difficult. Therefore, the pig was analysed here as a further species with a mammotypical flat embryonic disc. Using light and electron microscopy and in situ hybridisation for three key genes involved in early development (*sox17*, *nodal* and *brachyury*), two axial structures of early gastrulation in the pig were identified: (1) the anterior hypoblast (AHB) characterised by increased cellular height and density and by *sox17* expression, and (2) the early primitive streak characterised by a high pseudostratified epithelium with an almost continuous but unusually thick basement membrane, by localised epithelial–mesenchymal transition, and by *brachyury* expression in the epiblast. The stepwise appearance of these two axial structures was used to define three stages typical for mammals at the start of gastrulation. Intriguingly, the round shape and gradual posterior displacement of the APD in the pig appear to be species-specific (differing from all other mammals studied in detail to date) but correlate with ensuing specific primitive streak and extraembryonic mesoderm development. APD and, hence, the earliest axial structure presently known in the mammalian embryo may thus be functionally involved in shaping extraembryonic membranes and, possibly, the specific adult body form.

1. Introduction

One of the earliest axial structures in the mammalian embryo is an inconspicuous cellular differentiation at the anterior pole of the embryonic disc during the start of the gastrulation phase. Known as the anterior visceral endoderm (AVE) in the mouse (Rosenquist and Martin, 1995; Thomas and Beddington, 1996) or the anterior marginal crescent (AMC) in the rabbit (Viebahn et al., 1995a; cf. Kölliker, 1879), it is only transiently present (similar to other axial structures of gastrulation such as Hensen's node or the notochord) but draws its significance for development at least from the following three features: (1) AVE or AMC fixes two principal body axes (longitudinal and transversal) simultaneously by establishing structural cell shape changes at the (anterior) border of an embryonic disc, which is polarised along its dorsal–ventral (sagittal) axis only; (2) signaling potency residing in the AVE/AMC seems to be responsible for suppressing mesoderm formation and for inducing neuroectoderm or head identity in the epiblast (Knoetgen et al., 1999; Kimura et al., 2000; Idkowiak et al., 2004; Perea-Gomez et al., 2007; Egea et al., 2008), the latter function being suggestive of a Spemann type organiser (Beddington and Robertson, 1998; Hallonet et al., 2002; del Barco Barrantes et al., 2003, but s.a. Albazerchi and Stern, 2007); and (3) AVE/AMC cells appear to belong to the extraembryonic tissues that are shed after birth but, during early development, set up the basic body plan and the germ line using a complex expression pattern (“blue print”) of signaling molecules (cf. Ang and Constam, 2004; Georgiades and Rossant, 2006; Chuva de Sousa Lopes et al., 2007). As these features touch several essential processes of gastrulation and embryonic development as a whole, the anterior pole of the early mammalian embryo may have a developmental significance similar to the well-described primitive streak, with its morphogenetic potential and capacity to form mesoderm by epithelial–mesenchymal transition (EMT, cf. Voiculescu et al., 2007; Yang and Weinberg, 2008). Therefore and for the purpose of the present report, we subsume the different designations for the early anterior lower layer differentiation in

☆ Join the International Society for Differentiation (www.isdifferentiation.org).
* Corresponding author. Tel.: +49 551 39 7000; fax: +49 551 39 7043.
E-mail address: cviebah@gwdg.de (C. Viebahn).

0301-4681/$ - see front matter © 2009 International Society of Differentiation. Published by Elsevier Ltd. All rights reserved.
doi:10.1016/j.diff.2009.07.006

the mammalian embryo (see also Viebahn, 1999) under the heading of anterior pregastrulation differentiation (APD).

Mammalian species show surprising differences with respect to the topographical arrangement of the (relatively few) tissues involved in the start of gastrulation (see Behringer et al., 2006; Selwood and Johnson, 2006; Blomberg et al., 2008). As a consequence, size and mutual contact areas of (polar or mural) trophoblast, epiblast and hypoblast, for example, vary considerably; however, these characteristics correlate to some extent with the extraembryonic differentiation and the implantation schedule, which may start well ahead of gastrulation (mouse, higher primates and man) or may be delayed until the late gastrulation phase (rabbit) or advanced neurulation stages (ruminants, ungulates). Even within one of these groups the relative size of tissues may vary, too, the epiblast being rather large and "forced" into a cylinder shape (the "egg-cylinder") in the mouse (Tam and Gad, 2004), or being small and representing a flat disc as in higher primates including man. Mouse and rabbit are two popular laboratory species recently used for analysis of mammalian gastrulation as they represent opposite ends of this topography spectrum between egg-cylinder (mouse) and the mammotypical flat disc (rabbit). Importantly, both these species are amenable to experimentation (cf. Rossant and Tam, 2009; Reupke et al., 2009) during this phase of development, which, in mammals, has so far been impossible to observe directly in the secluded environment of the uterus. By comparison of results obtained in these two species, it can be assumed that general rules for the mechanisms running mammalian gastrulation may be derived. However, even if careful approximations are taken into account to project the complex topography and expression patterns of the rodent egg-cylinder onto the mammotypical embryonic disc (cf. Behringer et al., 2000), topographical and molecular results can be matched only partially between these two species (cf. Blomberg et al., 2008), making it impossible to decide which results may be generally applicable to mammals and which may be species-specific.

In search of general rules for axial differentiation at the start of mammalian gastrulation, the present study intends to establish the basis for using the pig as a third mammalian species in experimental gastrulation analysis. The pig has the mammotypical flat disc (Fléchon, 1978; Barends et al., 1989; Vejlsted et al., 2006) but a (late) implantation schedule (Heuser and Streeter, 1929; Patten, 1948; Perry and Rowlands, 1962) differing distinctly from that of the rabbit; the pig may therefore have to accommodate extraembryonic signaling for setting up the body plan in a yet again different topographical arrangement. Not least because of its late implantation, the pig seems also well suited for experimental analysis of this phase of development (Papaioannou and Ebert, 1988; Fléchon et al., 1995; Wianny et al., 1997). However, it is presently not clear how initial axial differentiation or early and pregastrulation stages can be defined in the pig, because a systematic comparison of standard dorsal (orthogonal) views of the embryonic disc is lacking at these early stages. Also, EMT which is the hallmark of mesoderm formation, has not been defined or correlated with the gross morphology of primitive streak formation (Fléchon et al., 2004). Therefore, high-resolution morphological analysis as well as in situ hybridisation for the expression of the axial differentiation and patterning genes *sox17* (cf. Pfister et al., 2007; Hassoun et al., 2009), *nodal* (cf. Lu and Robertson, 2004; Mesnard et al., 2006; Liguori et al., 2008) and the key mesoderm marker gene *brachyury* (Herrmann, 1991, cf. Arnold and Robertson, 2009) are applied here to closely spaced pre- and early gastrulation stages of the pig (between 8 and 10 days post conception, d.p.c.). In this way we find typical signs of APD in the hypoblast, mesoderm precursors in the epiblast and the EMT as the hallmark of initiating overt primitive streak formation. On the basis of these structural and molecular features three stages can be defined at the start of gastrulation, which may well be applicable to mammals in general and helps to clarify differences and similarities between mammals during this crucial period of development.

2. Methods

2.1. Animal tissues

Late pre pubertal gilts (Landrace × Large White, Institute of Animal Science and Behaviour, 31535 Mariensee, Germany) were stimulated using 5 ml equine Regumate® (2.2 mg/ml, Intervet, Unterschleißheim, Germany) per os once daily for 10–18 days and using 1500 IU pregnant mare serum gonadotropin (Integonan®, Intervet) i.m. 72 h prior to mating with Piétrain boars; on the day before starting the mating schedule, gilts were superovulated using 500 IU chorionic gonadotropin (Ovogest®, Intervet) i.v. Each gilt was mated (or artificially inseminated) twice, the first time 24 h after hCG treatment and a second time 36 or 48 h after hCG treatment. The time of the first mating or insemination was taken to be the time of conception from which embryonic age was calculated, i.e. embryos designated to be recovered at 8.0 days post conception (d.p.c.) had an embryonic age of minimally 7.0 and maximally 8.0 days. Uteri were removed after slaughter between 8.0 and 10.0 d.p.c. (2 litters with a total of 32 embryos at 8 d.p.c., 3 litters with a total of 70 embryos at 9 d.p.c., and 2 litters with a total of 61 embryos at 10 d.p.c.).

Uterine horns were flushed twice with 20 ml warm (37 °C) phosphate-buffered saline (PBS) containing 2% polyvinyl alcohol (PVA). For in situ hybridisation blastocysts were fixed in 4% paraformaldehyde (PFA) in phosphate buffer for 2–3 h at room temperature; after microdissection using iridectomy scissors and tungsten needles, specimens were dehydrated in a graded series of ethanol and frozen in 100% ethanol at −20 °C until used for in situ hybridisation. For high-resolution light and transmission electron-microscopical analysis, blastocysts were prefixed for 2–3 h in 1.5% PFA and 1.5% glutaraldehyde (GA) in phosphate buffer followed by microdissection as above, postfixation in 1% OsO_4 in phosphate buffer and embedding in Araldite® (Schwartz et al., 1984).

2.2. Morphological analysis

OsO_4-fixed embryos were photographed as whole -mounts from their ventral and dorsal sides prior to embedding in Araldite® (Plano, Wetzlar, Germany), i.e. while suspended in phosphate buffer, and again after embedding, for faithful topographical correlation of structures observed in the serial sections obtained in the following step. Complete series of semithin (1 μm) sections were cut from a total of 11 Araldite®-embedded embryos either in the transverse or in the sagittal plane (determined with the help of the whole-mount photographs) and stained with methylene blue (Schwartz et al., 1984). Peripheral tissue borders created in the blastocyst wall by microdissection were traced back to the edges of the stained semithin sections and used to define the angle and position of individual serial sections within the whole-mount photographs taken prior to and after embedding. At suitable intervals 70 nm sections were cut for transmission electron-microscopical analysis of regions defined to be of interest in semithin sections and dorsal views of whole blastocysts. If necessary, selected semithin sections were re-embedded in Araldite® (Viebahn et al., 1995b) and sectioned at 70 nm.