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Gastrulation and the establishment of the three germ layers in the early horse conceptus

Maria M.F. Gaivão*, Björn P.B. Rambags [1], Tom A.E. Stout

Department of Equine Sciences, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands

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ABSTRACT

Experimental studies and field surveys suggest that embryonic loss during the first 6 weeks of gestation is a common occurrence in the mare. During the first 2 weeks of development, a number of important cell differentiation events must occur to yield a viable embryo proper containing all three major germ layers (ectoderm, mesoderm, and endoderm). Because formation of the mesoderm and primitive streak are critical to the development of the embryo proper, but have not been described extensively in the horse, we examined tissue development and differentiation in early horse conceptuses using a combination of stereomicroscopy, light microscopy, and immunohistochemistry. Ingression of epiblast cells to form the mesoderm was first observed on day 12 after ovulation; by Day 18 the conceptus had completed a series of differentiation events and morphologic changes that yielded an embryo proper with a functional circulation. While mesoderm precursor cells were present from Day 12 after ovulation, vimentin expression was not detectable until Day 14, suggesting that initial differentiation of mesoderm from the epiblast in the horse is independent of this intermediate filament protein, a situation that contrasts with other domestic species. Development of the other major embryonic germ layers was similar to other species. For example, ectodermal cells expressed cytokeratins, and there was a clear demarcation in staining intensity between embryonic ectoderm and trophectoderm. Hypoblast showed clear α1-fetoprotein expression from as early as Day 10 after ovulation, and seemed to be the only source of α1-fetoprotein in the early conceptus.

1. Introduction

"Gastrulation" is the first major morphogenetic change that a developing embryo undergoes and refers to the stage at which the in-folding that subsequently gives rise to the digestive system and musculature can first be seen as a distinct cell layer within the embryo proper [1,2]. However, because not all mammalian embryos undergo an identical transformation, it has become more common to define gastrulation as the morphogenetic processes involved in the transition of a simple, unstructured ball of cells into a more complex, organized, and multilayered conceptus with distinguishable progenitors of all the major tissues of the adult organism: Endoderm, mesoderm, and ectoderm [1–3].

Gastrulation is a dynamic process in which the three germ layers are generated during a series of complex movements of individual cells or contiguous sheets of cells [3–5]. The first differentiation event during mammalian embryo development is divergence into trophectoderm and inner cell mass (ICM). The ICM cells subsequently differentiate into distinct internal and external layers to form a bilaminar embryonic disc. The internal layer of this disc is known as the hypoblast and is derived from cells that migrate from the ICM and proceed to line the blastocoel cavity, thereby completing a bilaminar yolk sac [6]. Although the hypoblast gives rise

* Corresponding author. Tel.: +35 1917814219
E-mail address: p3030@ulusofona.pt (M.M.F. Gaivão).
[1] Present address: uQinisa, Houten, The Netherlands.

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exclusively to extra-embryonic structures, it is thought to play an important role in embryo patterning [2,7]. The ICM layer above the hypoblast is known as the epiblast and is believed to contain all of the progenitor cells that eventually contribute to the embryo proper.

Gastrulation begins when an elongated thickening of epiblast develops into the primitive streak (PS), the future longitudinal body axis. Along the PS, epiblast cells migrate ("ingress") to the midline where they are transformed into mes-endoderm precursor cells, that is, cells capable of forming either mesoderm or endoderm, by epithelial–mesenchymal transformation, before migrating laterally where most will contribute to mesodermal organ formation. However, some mes-endoderm precursor cells are incorporated into the hypoblast where they form the definitive endoderm; the remaining epiblast cells never undergo ingression and instead differentiate into ectoderm [6,8–11]. After the mesoderm and endoderm precursors have migrated through the PS, the germ layers are patterned to generate specific tissues along the dorsoventral and anterior–posterior embryonic axes. The outer layer (ectoderm) gives rise to epidermis and neural tissues, the inner layer (endoderm) develops into the digestive tract and its associated organs (pancreas, liver) and the middle layer (mesoderm) gives rise to other organs (heart, kidney, gonads), connective tissue (bone, muscles, tendons), and blood cells [2].

The PS arises at the future posterior end of the embryo, where it appears as a faint groove along the midline of the bilaminar disc that defines the anterior–posterior (future head–tail) axis of the embryo. The PS then lengthens linearly toward the future anterior end of the embryo [2,11,12]. In general, endoderm is internalized before mesoderm, and the endodermal cells initially migrate individually. One of the most striking differences between mouse and either human or large domestic animal embryonic development is the mechanism of extra-embryonic mesoderm formation. In the mouse, mesoderm is derived exclusively from epiblast cells that ingress via the PS [13], whereas in man [13,14], pigs [15], cattle [16], sheep [17,18], and rabbits [19], precocious mesoderm is evident before the appearance of the PS. Intriguingly, the ingressing mesoderm cells are somehow directed differentially, some migrating laterally and anteriorly and others remaining on the midline. The cells that migrate laterally condense into three main rod and sheet-like structures on either side of the notochord: (1) The paraxial mesoderm, which lies immediately adjacent to and flanks the notochord, and develops into the somites; (2) the intermediate mesoderm, which lies just lateral to the paraxial mesoderm; and (3) the lateral plate mesoderm, a flattened sheet that forms the coelomic cavity by splitting into two layers, the splanchnopleuric mesoderm adjacent to the endoderm and the somatopleuric mesoderm adjacent to the ectoderm [6,10].

The onset of organogenesis is marked by the development of neural tissue from precursor cells derived from embryonic ectoderm. By the end of gastrulation, these neural precursor cells no longer respond to signals that induce alternative fates, and are thus committed to a neural lineage [20]. During neurulation, the neural precursor cells directly overlying the notochord and somites fold in and fuse to form the neural tube, whereas further cells from the neural folds ingress as the neural crest and subsequently differentiate in diverse ways to contribute to tissues such as mesenchyme, bones, and cartilage (particularly in the head), melanocytes, and the peripheral nervous system [21].

Research into mammalian embryology has concentrated on *Mus musculus*, the laboratory mouse. However, it is questionable whether the mouse is the most appropriate model for mammalian embryogenesis as a whole, given a number of striking morphologic differences between early rodent embryo development and that in other mammals. Early embryonic development in the horse seems to more closely resemble that in man and the large farm animal species and, in addition, the conceptus and developing embryo proper are relatively easy to visualize ultrasonographically (i.e., noninvasively) from early in development (Days 10 and 21, respectively). Moreover, intact conceptuses can be recovered relatively easily and atraumatically from the standing mare until at least Day 40 of gestation (i.e., before definitive placenta formation). In no other domestic species can entire conceptuses at such an advanced developmental stage be obtained from the uterus repeatedly, nonsurgically and free of maternal tissues with such ease. To date, however, there have been few studies of cell lineage segregation during early equine embryogenesis. These processes are of both scientific and practical interest. During the period before definitive placenta formation, numerous important events take place that differ markedly in character from those described for other animal species. Moreover, little is known about cell differentiation events during early equine embryo development.

The aim of this preliminary study was to use a combination of stereomicroscopic, histologic, and immunohistochemical techniques to examine embryonic development during a period (Days 10–18) expected to encompass gastrulation and the formation of both the embryo proper and the primitive (vitelline) circulation.

2. Material and methods

2.1. Animals

During the physiologic breeding season, 13 normally cycling Dutch Warmblood mares maintained at grass at Utrecht University's Department of Equine Sciences were used repeatedly as conceptus donors; all procedures were approved by the Animal Experiment Committee.

When they were in estrus, mares were teased three times a week using a vigorous stallion, and their reproductive tract was examined by transrectal palpation and ultrasonography, using a MyLabs 30 (Esoate, Maastricht, The Netherlands) ultrasound machine equipped with a 7.5-MHz linear array transducer. Once a dominant follicle(s) exceeding 35 mm in diameter was observed, mares were inseminated with 300×10^6 morphologically normal, progressively motile sperm from a single fertile stallion. Artificial insemination was repeated every other day until ovulation and, after the first artificial insemination, mares were examined daily to determine the day of ovulation (Day 0; as determined by the emptying of the preovulatory