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Ex vivo culture of pre-placental tissues reveals that the allantois is required for maintained expression of *Gcm1* and *Tpbpα*

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ABSTRACT

Introduction: Chorioallantoic fusion is essential for development of the labyrinth layer of the mouse placenta. However, events that occur after chorioallantoic attachment remain poorly described, partly due to difficulties of conducting *ex vivo* analysis of the placenta. Herein, we report conditions for *ex vivo* culture of the developing murine placenta.

Methods: Mesometrial halves of decidua containing pre-attachment chorions were cultured alone or with explants of allantoides from stage-matched controls and analyzed by confocal and immunofluorescence microscopy. Expression and levels of marker genes associated with specific placental cell types were measured by *in situ* hybridization and qRT-PCR, respectively.

Results: After 24 h (hr) of co-culture, a mosaic pattern of $eGFP^{+}$ and $eGFP^{-}$ cells were found when explants of pre-attachment chorions from $eGFP^{+}$ embryos were co-cultured with stage-matched allantoides from $eGFP^{-}$ embryos or vice versa. In addition, proliferation increased in the allantoic region and folds formed on the chorionic plate. PECAM positive cells derived from the allantois were found in the chorionic region. Levels of the SynT-II marker, *Gcm1*, significantly increased at 24 h, although expression of *Gcm1*, was only found in explants co-cultured with an allantois at 12 h and 24 h. In addition, though levels of *Tpbpα* was not altered by co-culture with an allantois, *Tpbpα* was only detected in explants co-cultured with an allantois for 24 h.

Discussion: Our data show that chorioallantoic fusion and events associated with initiation of labyrinth layer formation can be modeled *ex vivo*, and reveal a previously unsuspected requirement of chorioallantoic fusion for *Tpbpα* expression.

1. Introduction

The placenta is the interface for normal metabolic and gas exchange between the fetal and the maternal circulation, and is essential for rapid growth of mammalian embryos [1]. In mouse, the mature placenta consists of multiple cell types that are organized into three histologically distinct zones: the maternal decidua, the junctional zone, and the inner labyrinth layer. The labyrinth layer is a highly branched structure consisting of trophoblast-derived syncytiotrophoblast and giant cells which forms a trilaminar layer to separate fetal blood vessels and maternal sinuses. The junctional zone consists predominantly of spongiotrophoblast and glycogen cells that originate from the ectoplacental zone. Exchanges of nutrients, gas, and waste occur in the labyrinth layer, while the junctional zone provides support for placental growth and hormone production [2].

Formation of the labyrinth layer depends on chorioallantoic attachment and subsequent fusion of the chorion and allantois. Prior to chorioallantoic attachment, the allantois emerges as a bud of mesoderm cells from the posterior end of the embryos and grows towards the chorion. Trophoblast cells of the chorion undergo rapid proliferation which results in folding at the center of the chorionic plate and obliteration of the ectoplacental cavity [3]. Chorioallantoic attachment is mediated by interactions between extraembryonic mesoderm cells in the allantois and on the basal surface of the

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chorion; and occurs between embryonic day (E) 8.0 and E9.0 in embryos with 6 or more somite pairs (s) [4]. Recombination of allantois and chorion from embryos of asynchronous developmental stages showed that the chorion is competent to fuse with an allantois starting at the 1s stage, whereas the allantois exhibits maximal fusion in embryos with 3–5s [4,5].

During chorioallantoic fusion, the mesothelial layer of the chorion which mediates the initial contact between the chorion and allantois is thought to degenerate [5], and a subset of chorionic trophoblast cells downregulates expression of tight-junction proteins, such as zona occluden 1 (ZO1) [6]. On the other hand, the allantois spreads on the surface of the chorion to initiate the process of labyrinth layer formation [7]. The primary vascular plexus of the allantois, consisting of endothelial cells expressing *Pecam1*, *Vcam1*, and *Flk1* invades the chorion by sprouting angiogenesis [8,9]. In addition, chorionic trophoblasts that express the transcription factor *Gcm1* exit the cell cycle and initiate branching morphogenesis [10,11]. *Gcm1* expressing cells differentiate into one of the two syncytiotrophoblast cell-types, SynT-II, which separates allantois-derived embryonic blood vessels and maternal sinuses. In mutant embryos where chorioallantoic fusion fails to occur or in mice with loss of function mutations in *Gcm1*, branching morphogenesis is not initiated. In these mutants, both SynT-II and SynT-I cell types fail to differentiate and consequently the labyrinth layer of the placenta does not form [12–15].

In addition to chorioallantoic fusion, signals from the ectoplacental cone/spongiotrophoblast are also required for labyrinth layer development. Mutations in genes required for maintenance of spongiotrophoblast lead to abnormal labyrinth layer development, and subsequently results in embryonic death [16–18]. The specific contribution of the spongiotrophoblast to labyrinth layer formation is not clear, although it is postulated that these cells may provide signals or structural support important for labyrinth layer formation [19].

E*x vivo* organ cultures are widely used and provide insights into developmental processes such as kidney and lung morphogenesis [20–22]. A number of *ex vivo* placental models have been described, but are not well characterized. *Ex vivo* culture of embryos and their associated pre-placental tissues (ectoplacental cone, chorion and allantois) after chorioallantoic fusion supports syncytiotrophoblast differentiation and branching morphogenesis in static cultures [3]. However, the ectoplacental cleft remains open and levels of *Syncytin A*, a marker of SynT-I decreases within 12 h [23]. In *ex vivo* cultures of pre-placental tissues after chorioallantoic fusion and without an embryo, the allantoic mesoderm degenerates and trophoblast differentiation is perturbed, as indicated by decreased expression of *Gcm1* and *Syncytin A*. Although expression of *Tpbpα*, a spongiotrophoblast marker, significantly increased in these *ex vivo* cultures, explants of pre-placental tissues post chorioallantoic fusion do not mimic the molecular differentiation events normally found *in vivo* [23]. Furthermore, explants of ectoplacental cone and chorion before chorioallantoic fusion showed that *Gcm1* expression was only found if these explants are co-cultured with an allantois such that there is physical contact between the chorion and allantois [24]. Although, additional molecular and histological analysis of these explants were not performed, this finding suggests that explants of pre-placental tissues before chorioallantoic fusion may be a better model for studying the early events associated with placental development [24].

We set out to determine if pre-attachment ectoplacental cones (EPCs) and chorions left in their associated decidua can be cultured with pre-attachment allantoides to mimic some of the events associated with early morphogenesis of the labyrinth layer. Herein, we report conditions for an *ex vivo* recombination system that recapitulate many of the changes associated with chorioallantoic fusion including: molecular changes, such as increased *Gcm1* expression and maintained expression of *Tpbpα*, morphogenic changes and trophoblast differentiation, including mixing of chorionic and allantoic cells and initiation of branching morphogenesis. We propose that this *ex-vivo* model can be used to study chorionic and allantoic-specific contribution of genes required for early placental development.

2. Material and methods

2.1. Animals

All procedures and experiments were performed according to the guidelines of the Canadian Council on Animal Care and approved by the Animal Care Committee of the Montreal Children's Hospital. CD1 (*Charles River*) mice were used to collect wild type tissues, and Tg(HIST1H2BB/EGFP)1Pa [25] - a transgenic line on a mixed genetic background that ubiquitously expresses an H2B-eGFP fusion protein in the nucleus - was used for collecting eGFP + tissues. Tg(HIST1H2BB/EGFP)1Pa was a kind gift from Dr. K. Hadjantonakis. Retired stud male rats were purchased from *The Jackson Laboratory* for serum collection.

2.2. Rat serum collection

Blood was collected from the dorsal aorta of anesthetized rats. Rat serum was obtained after immediate centrifugation of the blood and stored at −80 °C until preparation of the culture medium.

2.3. Isolation of allantoides and chorions for explant cultures

Female and male mice were mated overnight, and the presence of a plug the following morning was noted as E0.5. Pregnant females were euthanized at E8.0. On the day of dissection, decidua was removed from the uterus (Fig. 1A). To remove embryos, an incision was made on the anti-mesometrial side of each decidua to remove the overlying tissue (Fig. 1B). After the anti-mesometrial portion of the decidua was removed, EGFP $^{+}$ embryos were distinguished from eGFP^{-} embryos under a stereo microscope with a GFP filter. Fine forceps were used to separate embryos from their associated yolk sacs (Fig. 1B′). Embryos of 3–5s did not have chorioallantoic attachment and were easily separated from their extraembryonic tissues. The full allantois was cut from the tail-bud with a pair of fine forceps (Fig. 1C′). All dissections were performed in 1xPBS. Tissues were either processed for explant cultures as described below (2.4) or collected for day 0. Decidua/EPC/chorion samples collected at day 0 were individually fixed in 4% PFA overnight for immunohistochemistry or *in situ* hybridization. 3 pools of two chorion-only explants or two chorion and allantois explants were stored in Trizol (Invitrogen) and used for qRT-PCR.

2.4. Explant of decidua/EPC/chorion or decidua/EPC/chorion with allantoides

For decidua/EPC/chorion only explants, samples were placed with the distal side containing the chorionic plate up, in 500 μl of medium in 24-well tissue culture plates (Falcon) (Fig. 1D). For decidua/EPC/chorion and allantois explants, eGFP^{+} or negative decidua/EPC/chorion were placed as described above and a single eGFP^{-} or positive allantoides were placed on top of the chorion, at an approximately 45-degree angle (Fig. 1D′). Due to the convex morphology of the chorionic plate, the allantois remains associated with the chorion and can then be moved to 24-well tissue culture plates (Falcon) with 500 μl of medium for culture (Fig. 1D′).