Advanced Review

The mammalian blastocyst

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The blastocyst is a mammalian invention that carries the embryo from cleavage to gastrulation. For such a simple structure, it exhibits remarkable diversity in its mode of formation, morphology, longevity, and intimacy with the uterine endometrium. This review explores this diversity in the light of the evolution of viviparity, comparing the three main groups of mammals: monotremes, marsupials, and eutherians. The principal drivers in blastocyst evolution were loss of yolk coupled with evolution of the placenta. An important outcome of blastocyst development is differentiation of two extraembryonic lineages (trophoblast and hypoblast) that contribute to the placenta. While in many species trophoblast segregation is often coupled with blastocyst formation, in marsupials and at least some Afrotherians, these events do not coincide. Thus, many questions regarding the conservation of molecular mechanisms controlling these events are of great interest but currently unresolved.

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INTRODUCTION: MORPHOLOGICAL DIVERSITY OF MAMMALIAN BLASTOCYSTS

The blastocyst is an early stage of development that combines two key features of mammals: the expansion of extraembryonic cell lineages that contribute to placental function and the maintenance of a fluid-filled cavity that has largely or entirely (depending on species) replaced the yolk of their oviparous ancestors. Although morphologically similar, the blastocyst is distinct from the blastulae of invertebrate animals that do not set aside extraembryonic tissues during development, because much or all of its outer epithelium gives rise to extraembryonic ectoderm. In mammals, unlike in most other vertebrates, the extraembryonic ectoderm is specially adapted for placental function by taking up nutrients from the mother, and is called the *trophoblast*. This name was initially coined in 1889 by Hubrecht[1] to refer to the extraembryonic ectoderm of only marsupials and eutherians, but as monotremes have also been recognized to develop a functional placenta,[2] it is appropriate to extend its usage to all mammals.

The trophoblast is the first specialized cell lineage to form. It segregates early in development from another population of cells called the *pluriblast*,[3] which is fated to form all other cell lineages. In eutherians, the pluriblast emerges as a cluster of cells at one end of the blastocyst cavity and is entirely enveloped by the early trophoblast epithelium (often called *trophectoderm*). The eutherian pluriblast is thus conventionally termed the *inner cell mass* (ICM). In marsupials and monotremes, the pluriblast is not enveloped by the trophoblast but is instead part of a continuous epithelium that forms a unilaminar blastocyst. The pluriblast subsequently segregates into *hypoblast* (an extraembryonic endodermal lineage) and *epiblast*, which gives rise to all three embryonic germ layers (ectoderm, mesoderm, and endoderm), extraembryonic mesoderm, and germ cells.

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The morphology of the mammalian blastocyst is extraordinarily diverse among species (Figure 1). This diversity is consistent with the hourglass model of development,[4,5] whereby an evolutionarily conserved phylotypic stage (the 'pharyngula' stage in vertebrates) is both preceded and succeeded by phases that are more divergent in both morphology and gene expression. Part of the diversity in blastocyst morphology is readily explained by the amount of deutoplasm (secondary cytoplasm, including yolk bodies) present in the zygote, especially when comparing the three major groups of mammals—monotremes, marsupials, and eutherians. Further diversity within eutherians can also be explained by the mode and timing of placentation.

All mammals develop a placenta, which can be defined as a transient extraembryonic organ that facilitates nutrient exchange between the mother and fetus.[6] In monotremes, placental tissues never come into direct contact with maternal tissues. Nutrients instead diffuse through the noncellular conceptus coats that are formed from maternal secretions and persist until oviposition at around the 20-somite stage.[7] In marsupials, similar conceptus coats initially prevent placental attachment but break down approximately two-thirds of the way through pregnancy. Subsequent placental attachment to the endometrium is superficial in most marsupials, but in some species (e.g., bandicoots) it is highly invasive. In eutherians, implantation can occur early (e.g., rodents) or late (e.g., pigs) in development and is often very invasive.

The beginning of the blastocyst stage is readily defined by completion of a functional outer epithelium that is capable of fluid transport and thus blastocyst expansion. The capacity for expansion of total conceptus volume is a key feature of mammalian development and is inherently associated with an evolutionary loss of yolk (Figure 2). The yolk mass of birds and reptiles has an important infrastructural role and its loss in mammals is compensated by generation of a blastocyst cavity.[8] The end of the blastocyst stage is less well defined because there is a large amount of variation among species in the timing of placental attachment relative to the developmental stage of the embryo proper. Depending on the species, placental attachment occurs either shortly after hypoblast formation, after gastrulation, after somitogenesis, or not at all. Here, we define the end point of the blastocyst stage as the time at which the conceptus has some initial features of asymmetry that indicate later anteroposterior axis formation. Later stages of the conceptus that precede implantation are usually referred to as *chorionic vesicles*.[9]

BLASTOCYST FORMATION

Monotremes

Considerable variation exists in the mode of blastocyst formation among mammals, and much can be learned by examining these modes in all of the three major mammalian groups: monotremes, marsupials, and eutherians. Monotremes, which include the

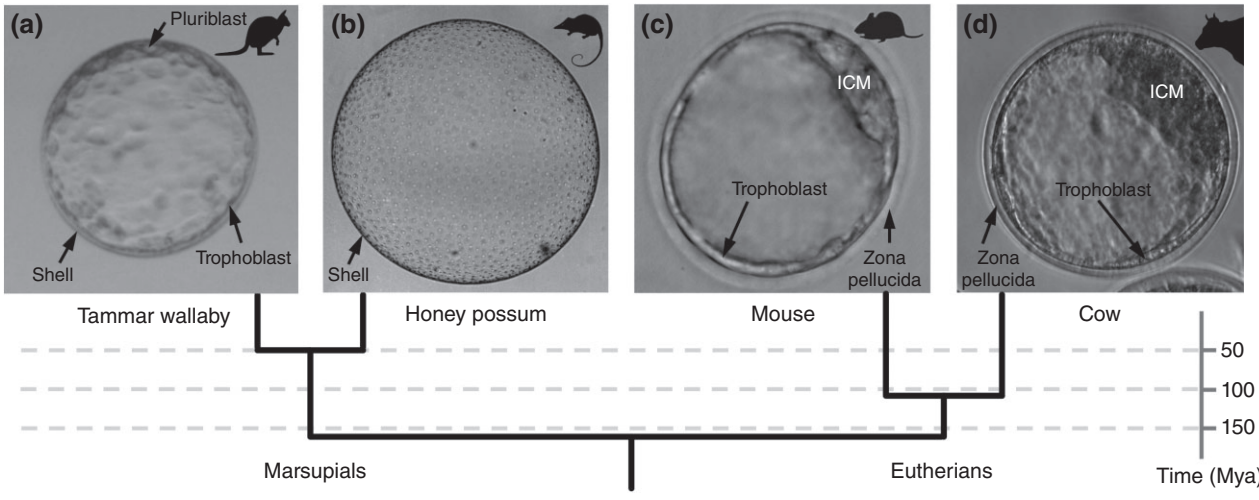

FIGURE 1 | Marsupial (a, b) and eutherian (c, d) blastocysts. (a) Day 9 (after removal of pouch young) blastocyst of the tammar wallaby. Note the absence of an inner cell mass. The attenuated mucoid coat and zona pellucida (not visible) lie between the blastocyst epithelium and the shell. (b) 2000-cell diapausing blastocyst of the honey possum. (c) Embryonic day 3.5 mouse blastocyst. (d) Day 8 bovine blastocyst (image courtesy of Marcelo D. Goissis and Jose Cibelli, Michigan State University).

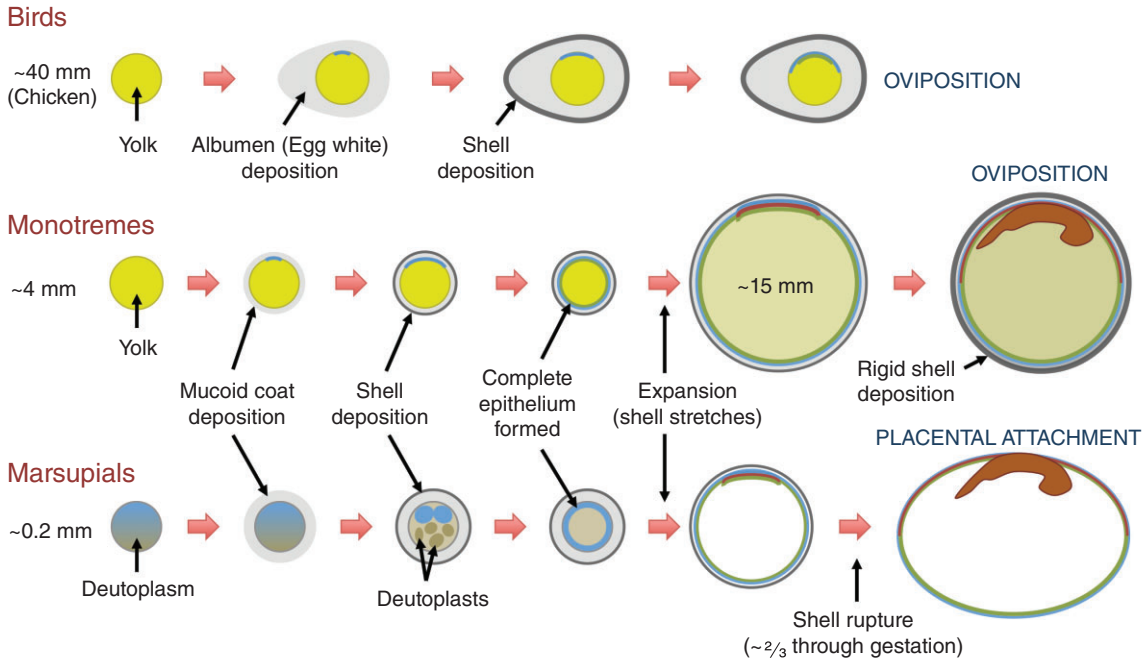

FIGURE 2 | Comparison of the relationship between yolk/deutoplasm content, coat deposition, and conceptus expansion in birds, monotremes, and marsupials.

platypus and several species of echidna, constitute an order within the subclass Prototheria, which is sister to the subclass Theria that includes marsupials and eutherians. Monotremes retain many ancestral features—including oviparity and their mode of embryonic development—that provide clues to the evolution of mammals from their therapsid reptile ancestors. Although monotremes are oviparous, the conceptus develops within the female reproductive tract until the somite stage, when it becomes encapsulated by a leathery shell before oviposition. At ovulation, monotreme ova are around 3–4 mm in diameter, much larger than those of therian mammals but also considerably smaller than the approximately 15–17 mm diameter of the laid egg (Figure 2). The majority of the monotreme ovum's cytoplasm consists of yolk that is at least partly homologous to the yolk of nonmammalian vertebrates. Most oviparous tetrapod genomes contain orthologues of three genes that, respectively, encode the major yolk proteins vitellogenin-1 (*VIT1*), -2 (*VIT2*), and -3 (*VIT3*), and the platypus genome contains at least one vitellogenin gene that appears to be functional.[10]

The early development of monotremes demonstrates many interesting features that suggest a transitional state in the evolution of mammalian blastocyst formation. Since the original publication by Caldwell,[11] most of our knowledge of monotreme development comes from early studies by Flynn and Hill,[12,13] with further analysis and interpretation by more recent authors.[14,15] The primary oocyte is highly polarized in the position of the germinal vesicle relative to the yolk and this polarity is maintained after fertilization and during subsequent development. As in birds and reptiles, cleavage is meroblastic and the associated asymmetry specifies an embryonic–abembryonic axis corresponding to the future dorsoventral axis of the embryo. Two populations of cells emerge at around the 16-cell stage: central cells and marginal cells. The latter give rise to 'vitellocytes,' which later fuse with each other to form a syncytial 'germ ring' surrounding a central, initially multilayered, blastoderm. As the blastoderm expands in area (while initially thinning until unilaminar), the germ ring also progressively extends toward the abembryonic pole while adhering to the inner surface of the zona pellucida, and eventually completely encloses the yolk. By that stage, the blastoderm has already produced a hypoblast layer; thus, the blastocyst is already bilaminar by the time it is fully formed and shortly before gastrulation.

Marsupials

Marsupial ova are typically around 200 μm in diameter, substantially smaller than those of monotremes but slightly larger than those of eutherians. They

contain a large number of vesicles whose contents are eliminated into the extracellular space during cleavage. Depending on the species, a large quantity of cytoplasm is also eliminated during the first division in the form of one or more large, membrane-bounded masses often called 'yolk masses.' Although marsupial ova are often described as 'yolky,' a nutritive role for their ooplasmic inclusions is unclear. Analysis of marsupial genomes shows at least that the genes encoding vitellogenin proteins are no longer functional.[10,16] The vast majority of the eliminated cytoplasm consists of translucent vesicles that ultimately contribute to a space-filling extracellular matrix.[17–19] Thus, 'deutoplasm' is the preferred term for this secondary cytoplasm because it makes no assumption regarding its role, while the yolk masses may accordingly be termed 'deutoplasts.'

In the zygotes of most marsupials, the position of the deutoplasm is overtly polarized relative to the pronuclei. Although holoblastic, the first division retains some features of meroblastic cleavage in its elimination of deutoplasm. The positions and properties of the blastomeres during early cleavage define an embryonic–abembryonic axis analogous to that in monotremes, birds, and reptiles. Blastomeres adhere to the inner surface of the zona pellucida and are initially concentrated in the embryonic hemisphere. While establishing cell–cell contacts to form a partial epithelium, they continue to divide and spread toward the abembryonic hemisphere, eventually forming a complete unilaminar blastocyst that proceeds to expand.

Some variation exists among marsupials regarding the mode of blastocyst formation (reviewed in Ref 20). Although information is incomplete for many taxa, general features characterizing some major groups are shown in Figure 3. The best-studied species represent three orders: the Dasyuromorphia [including especially the stripe-faced dunnart (*Sminthopsis macroura*)], the Diprotodontia [including the brushtail possum (*Trichosurus vulpecula*) and the tammar wallaby (*Macropus eugenii*)], and the Didelphimorphia [including the gray short-tailed opposum (*Monodelphis domestica*) and the Virginian opossum (*Didelphis virginiana*)]. In the Diprotodontia, epithelialization is precocious and blastomeres closely associate with one another at the embryonic pole as early as the two-cell stage. In the Dasyuromorphia, blastomeres are still separate by the eight-cell stage and form a ring or tier of cells adhering to the inner surface of the zona pellucida. The fourth round of latitudinal divisions results in two tiers of cells: one lying toward the embryonic pole and one lying toward the abembryonic pole. With subsequent cell–cell adhesion, a nascent blastocyst epithelium expands and covers first the embryonic pole and later the abembryonic pole. Cleavage in dasyurids is highly regular compared with other marsupials. A further feature is the production of a single large deutoplast that persists until well after blastocyst formation, unlike in other marsupials in which deutoplasts are usually multiple and rapidly fragment. Among the Didelphimorphia, very early studies of the Virginian opossum reported a lack of conceptus polarity in the zygote and early cleavage stages, although some polarity was evident by the eight-cell stage.[21–23] However, the gray short-tailed opossum, which is in the same family as the Virginian opossum, exhibits overt conceptus polarity not dissimilar to that in dasyurids.[24–26] It is most likely that early conceptus polarity in marsupials is a basal trait and it is possible that the early studies of *D. virginiana* overlooked certain polarity features due to histological methods and/or orientation of conceptuses before sectioning. A reexamination of new specimens would certainly be of value.

Eutherians

The most notable feature distinguishing eutherian blastocysts from those of marsupials is that the pluriblast takes the form of an ICM entirely enveloped by the trophoblast. By far the most studied species for blastocyst formation is the mouse. As for other eutherians, mouse ova are smaller than those of marsupials and contain negligible deutoplasm. Unlike in marsupials, the conceptus lacks overt polarity in both the position of the pronuclei after fertilization and during cleavage. Blastomeres do not adhere to the zona pellucida but instead adhere only to each other.

When the conceptus reaches the eight-cell stage, each blastomere starts to flatten its membrane against its neighbors, increasing the area of cell–cell contact.[27] It is then referred to as a *morula*—a structure unique to eutherians. In the mouse conceptus, compaction is the first morphological sign of differentiation by the formation of a polarized epithelium-like layer of cells, important for blastocyst cavitation. In humans, compaction of the morula is used as an indication of viability during *in vitro* culture.[28] The smoothening of the surface of the embryo is associated with an increase in the intercellular adhesion mediated by E-cadherin (encoded by *Cdh1*). Mouse embryos show compaction defects following *Cdh1* deletion or treatment with antibodies against E-cadherin.[29–34] E-cadherin-dependent filopodia were observed in intact mouse embryos injected with E-cadherin fused to GFP. During compaction, E-

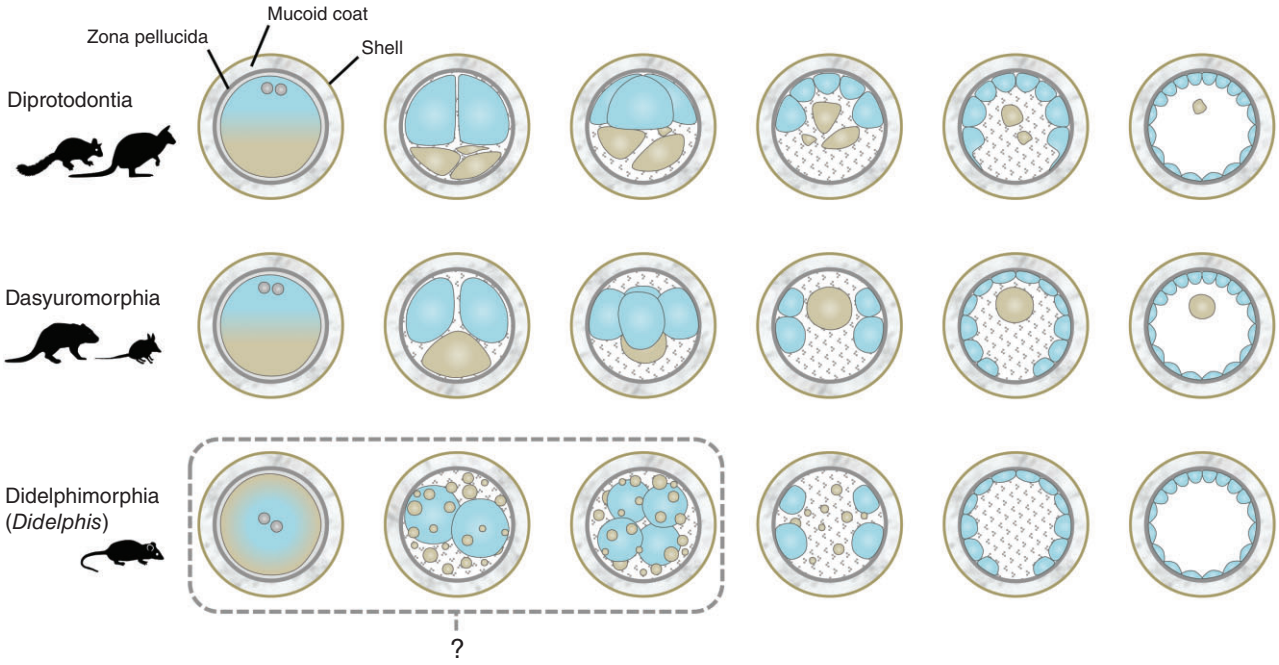

FIGURE 3 | Comparison of different modes of blastocyst formation among marsupials. All species exhibit early cell–zona adhesion that precedes cell–cell adhesion, which results in the formation of a unilaminar blastocyst. Variation occurs mainly in the degree of conceptus polarity, the number and size of deutoplasts, and the degree to which distinct populations of putative pluriblast and trophoblast precursors can be distinguished. In Didelphis virginiana, it is possible that early conceptus polarity (stages indicated by the boxed area) was overlooked in early studies, since it has only more recently been recognized in another didelphid, *Monodelphis domestica*.

cadherin–GFP was present throughout the cell membrane and enriched at adherens junctions, forming filopodium-like cell protrusions.[35]

Compaction should not be understood as an irreversible event, because decompaction and recompaction must occur during the ongoing cell divisions.[27,35–37] In the mouse, compaction occurs during two-cell cycles before blastocyst formation. In the pig embryo, the decompaction periods are longer and well defined,[37] being observed during the 16- to 32-cell transition. In cows, compaction starts at the 16-cell stage and the morula is completely compacted at the 32-cell stage,[36] similar to that which occurs in pig embryos.

Concomitant with compaction, individual blastomeres undergo apical–basal polarization.[27] Cell polarity is characterized by cell morphology, enrichment of microvilli and glycoproteins in the apical domain and by the localized expression of the PAR complex proteins.[27,38] After compaction and polarized cell divisions, most eutherian embryos, including in mouse and human, generate a morula with an outer polarized epithelium and an enclosed group of apolar inner cells. This has led to the polarization hypothesis[39] whereby asymmetric divisions can result in some daughter cells inheriting the apical pole and remaining at the embryo surface to form the trophoblast, while daughters not inheriting the pole would become enclosed, apolar, ICM precursors. Recent detailed analysis of cell behavior during divisions of polarized cells suggests that this is a too simple model and that there is dynamic repositioning of cells during the divisions leading up to blastocyst formation.[40–42] Cavitation begins at the late morula stage in these species and leads to the formation of an inner cavity and the positioning of the ICM at one pole of the cavity.

Mouse blastocyst formation starts at around the 32-cell stage, when outer cells of the morula form a fully functional epithelium. The trophoblast in the blastocyst is the first epithelium to be formed after fertilization. The presence of Na^+/K^+ ATPases and aquaporins ensures channeling of ions and water, allowing the formation of a fluid-filled cavity, denominated blastocyst cavity.[43] Once the cavity has appeared, the conceptus is referred to as a blastocyst. The function and composition of the blastocyst cavity, bathing both the trophoblast and ICM in eutherians, and the pluriblast and trophoblast in marsupials, remains poorly understood. It was first suggested that it supports cell migration during gastrulation, but most recent studies investigated a

possible direct role in supporting blastocyst development.[44,45] A proteomic analysis by mass spectrometry from *in vitro* produced surplus human blastocysts identified 286 proteins in the blastocoel fluid[44] being several heat-shock, ZP, vitamins, and vitamin-binding and ciliary proteins detected among others. A similar study was also conducted using bovine blastocysts, and the comparison of both datasets provided a list of nine proteins to be unique to the cow.[45] However, not one of them has any known relation to pluripotency or early development.

In some eutherians, cavitation precedes the formation of an inner cell population (Figure 4). Thus, in the elephant shrew there is no morula with inside cells as cavitation has already occurred during the four-cell stage.[46] The resulting unilaminar blastocyst expands until it has around 120 cells, when inner cells begin to be generated by divisions perpendicular to the blastocyst surface. These divisions apparently occur in random cells located anywhere within the blastocyst epithelium, suggesting that all cells of the unilaminar blastocyst are totipotent. Subsequently, inner cells extend long pseudopodium-like processes into the cavity to contact each other and eventually coalesce into an asymmetrically positioned ICM. Similarly in the tenrec, a unilaminar blastocyst forms by at least as early as the 16-cell stage[47,48] but, in contrast to the elephant shrew, inner cell generation (from around the 100-cell stage) is restricted to one pole of the blastocyst. According to these authors, conceptus polarity with respect to differences in cell size is evident during early cleavage and was assumed to correlate with the site of later inner cell generation.

Elephant shrews and tenrecs both belong to the superorder Afrotheria, which includes elephants, hyraxes, aardvarks, dugongs, and manatees and is distantly related to the Laurasiatheria and Euarchontoglires superorders that contain the majority of extant eutherian species. The presence of a unilaminar blastocyst in elephant shrews and tenrecs suggests that this may be a basal eutherian trait as it also occurs in marsupials, albeit via an altered mechanism. Unfortunately, early cleavage stages have not been described for any other Afrotherian species. Xenarthra (which includes sloths, anteaters, and armadillos) has often been considered the most basal eutherian superorder, although more recent evidence places it as a sister group to Afrotheria.[49,50] Information on Xenarthran blastocyst formation is similarly lacking. Development of the nine-banded armadillo has attracted much attention because implantation of

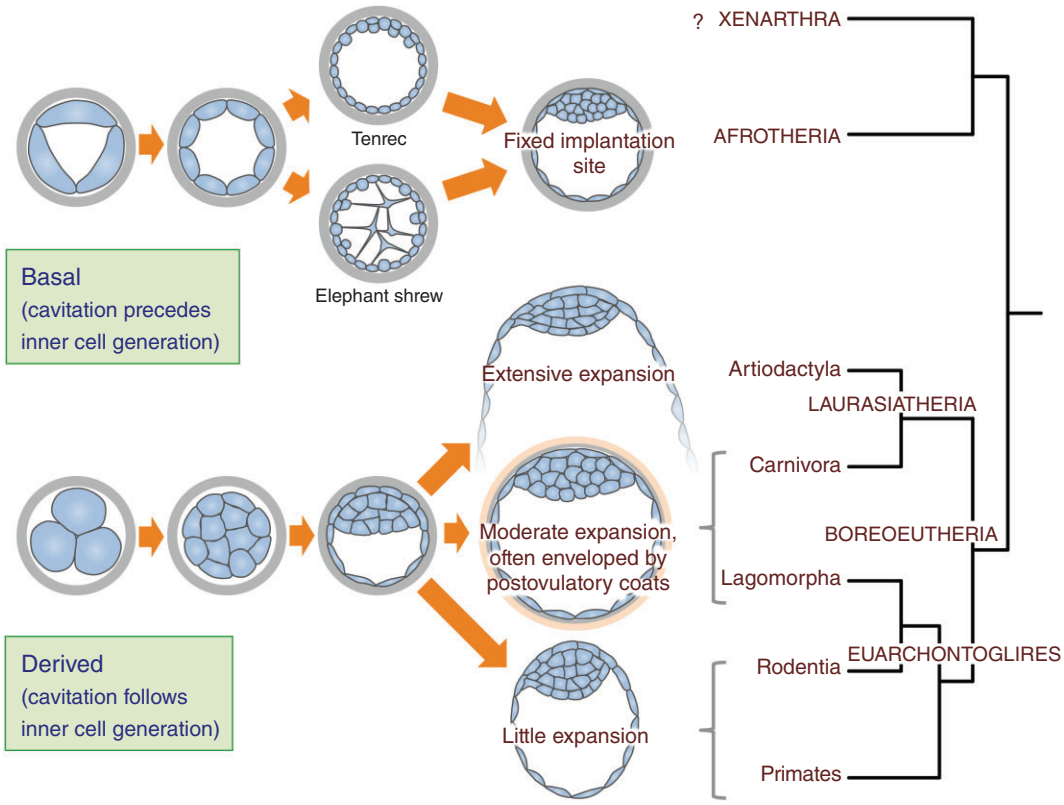

FIGURE 4 | Comparison of different modes of blastocyst formation among eutherians.

a single blastocyst normally results in the formation of four separate body axes and hence four genetically identical quadruplets.[51] In the only known report of early cleavage stages of an armadillo, Hamlett[52] made some curious observations. Although no cavity was reported for the 4-cell, 10-cell, and 20-cell stages examined, a small but distinct cavity is visible in one photographed section of the 10-cell stage. Thus, it is possible that armadillos also form a precocious unilaminar blastocyst and that the cavities of the specimens examined by Hamlett were largely obscured due to processing for histology. Curiously, Hamlett also reported large quantities of deutoplasm eliminated at one pole of the early cleavage stages, reminiscent of marsupial deutoplasmolysis. Further investigations are certainly warranted.

Although these Afrotherian and possibly Xenarthran species share similarities with marsupials in having a unilaminar blastocyst stage, they differ in that once the pluriblast (ICM) is specified, it does not occupy a superficial position within the blastocyst epithelium. These differences suggest that cavitation and ICM specification are separable events, with important implications for the possible mechanisms of cell fate specification. It is possible that in eutherians the polar trophoblast (overlying the ICM) evolved in concert with early implantation to prevent direct contact between the epiblast and maternal tissues. This is prevented in marsupials because amnion formation occurs well before shell breakdown allows placental attachment. In some eutherians (e.g., cow, pig, horse, and rabbit), the polar trophoblast (Rauber's layer) is secondarily lost so that the epiblast occupies a superficial position, but other mechanisms may have evolved later to enable tolerance of early epiblast–endometrial proximity. In the horse and rabbit, for instance, extracellular coats prevent early epiblast–endometrial contact. An alternative view is that polar trophoblast is essential in all eutherians only until the epiblast epithelializes, after which the latter can take on the role in some species of maintaining a seal for blastocyst expansion. Clearly in marsupials, all pluriblast cells have this essential epithelial function from their earliest formation.

MECHANISMS OF CELL LINEAGE SPECIFICATION

Trophoblast–Pluriblast

Eutherians

Two complementary models that describe the specification of trophoblast and ICM (pluriblast) lineages in the mouse have been proposed. The inside–outside model[53] proposed that positional cues are essential for specifying lineage identity. The cell polarity model[39] proposed that cell polarity is essential for specifying trophoblast. Cell position and cell polarity are considered to be mutually reinforcing properties.[54] Thus, by the time of blastocyst formation, outer cells retain their position and continue to epithelialize, while inner cells remain nonpolar and cannot contribute to the trophoblast epithelium. However, even through to mid-blastocyst stages, blastomeres retain the potential to change fate in the context of experimental manipulations.[31,55–59]

The first evidence for a molecular pathway directing differential gene expression between trophoblast and ICM came with the observation that *Tead4* null mutant embryos do not upregulate trophoblast-specific markers and fail to cavitate.[60] TEAD4 is a transcription factor whose activity depends on the co-activators YAP or its close relative WWTR1 (also called TAZ). YAP and WWTR1 are targets of the Hippo signaling pathway, which was originally identified in *Drosophila* and is important in regulating organ size and cell proliferation. In the canonical mammalian Hippo pathway, activity of YAP and WWTR1 is regulated by the upstream kinases LATS1/LATS2, which phosphorylate YAP/WWTR1 and prevent their nuclear entry by targeting them for degradation. Accordingly, overexpression of LATS2 causes suppression of CDX2 expression, while *Lats1*$^{-/-}$;*Lats2*$^{-/-}$ double mutants or overexpression of a dominant-negative LATS2 causes CDX2 expression in inside cells.[61] Active Hippo signaling in inside cells to suppress YAP/WWTR1 nuclear localization is linked to cell polarity. Two proteins—NF2 and AMOT—have thus far been implicated in translating cell polarity cues to regulation of Hippo signaling.[62–64] Therefore, TEAD4 partners with YAP and WWTR1 to promote CDX2 expression in trophoblast cells. Little is known of the importance of Hippo signaling in early development of other mammals. It is possible that this mechanism is conserved in cattle as TEAD4 is expressed in the morula, although later its relative expression along with YAP is similar between the ICM and trophoblast.[65,66]

In the mouse, trophoblast and ICM become committed by the expression of CDX2 and POU5F1 (also called OCT4), respectively, at the late blastocyst stage[67,68] (Figure 5). *POU5F1* is expressed throughout early mouse development until the blastocyst stage, when it becomes restricted to the ICM,[69] whereas CDX2 expression is restricted to outside cells of the late morula/early blastocyst. However,

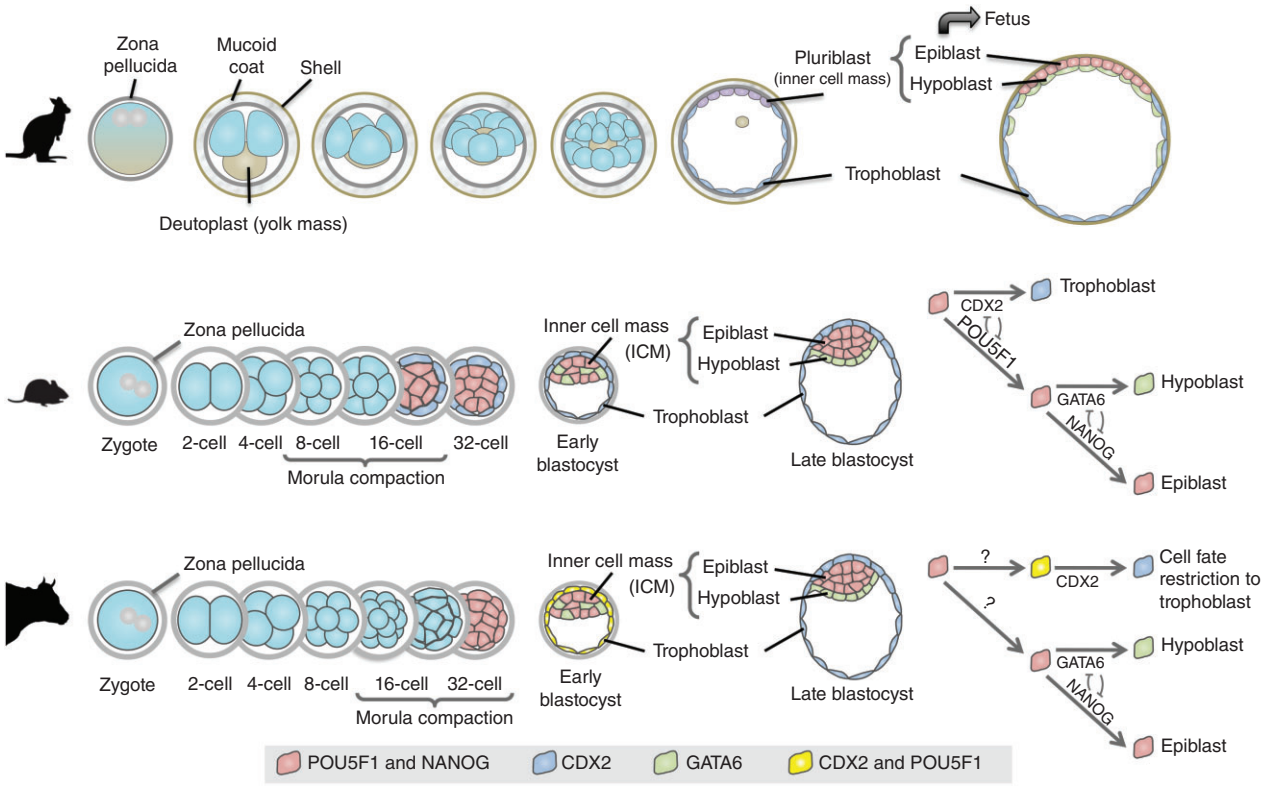

FIGURE 5 | Comparison of early cell lineage specification between tammar, mouse, and cow.

the same sequence of gene expression is not observed in bovine (Figure 5) and human embryos at the equivalent stage, suggesting that there may be differences in at least the timing of lineage commitment and possibly of the molecular mechanisms involved. CDX2 is required later by the bovine embryo and does not repress *POU5F1* expression.[70] CDX2 protein is restricted to the trophoblast in cow blastocysts,[71] but POU5F1 is not restricted to the ICM of blastocysts until well beyond the expanded blastocyst stage in humans, rhesus monkeys, pigs, and cows.[72–77] The co-expression of *CDX2* and *POU5F1* in expanded blastocysts suggests a late commitment of the trophoblast fate in these species. Outer cells recovered from decavitated and decompacted early human blastocysts and placed inside an empty zona pellucida formed blastocysts with a trophoblast and an ICM, verified by the respective positive staining for HLA-G and NANOG.[78] Similar to what was observed for cows,[70,79] *CDX2* expression follows human blastocyst formation, suggesting its expression is not required for differentiation of the outer epithelial layer and for cavity formation.[80] In addition, *CDX2* is not involved in the restriction of *SOX2* expression to the ICM of mouse and bovine blastocysts. Recent studies suggest that *SOX2* is a unique early ICM marker in mice and cows as it starts to be expressed in ICM–progenitor cells at the 16-cell stage, and its expression is restricted to ICM cells in the early blastocyst stage.[81,82] It is worth noting that even in the mouse, where CDX2 and POU5F1 expression are lineage-restricted by the mid-blastocyst stage, loss of CDX2 does not lead to failure of initial outer epithelial formation and cavity formation.[68] Thus, the events of polarization, cavity formation, and trophoblast and ICM cell fate specification are related but separable events across different eutherian species.

Marsupials

Early conceptus polarity in marsupials has been proposed to provide a mechanism for segregation of pluriblast and trophoblast lineages.[20] Thus, during early cleavage, putative pluriblast progenitors are identified by their proximity to the embryonic pole, while putative trophoblast progenitors occupy a more peripheral position within the developing epithelium prior to complete blastocyst formation (Figure 3). Although no experimental studies have been performed to demonstrate the fate of these respective blastomere populations and in many species cells become morphologically indistinguishable

during early unilaminar blastocyst stages, it is generally assumed that underlying differences between cells are retained during this phase and mark later cell fate. After a period of blastocyst expansion, the definitive pluriblast is distinct within a restricted region of the unilaminar epithelium and closely coincides with the onset of hypoblast formation (see below).

Marsupial genomes contain genes encoding orthologues of all the known key lineage-specific transcription factors involved in trophoblast–ICM segregation in the mouse, such as POU5F1 (OCT4), SOX2, NANOG, CDX2, and GATA3. Additionally, marsupial genomes contain *POU5F3* (previously called *POU2*), an ancient paralogue of *POU5F1* found in many other vertebrate genomes.[83–85] Only one study of the tammar wallaby has thus far attempted to identify marsupial pluriblast and trophoblast populations based on these molecular markers.[86] In the tammar, all cells in the unilaminar blastocyst appear identical until an embryonic disc appears at around days 9–10 after removal of pouch young (RPY) to reactivate from diapause. None of the markers showed differential expression either during cleavage or in diapausing blastocysts, suggesting that cells have not committed to distinct fates. Thus, it appears that if early conceptus polarity provides a cue for later segregation of pluriblast and trophoblast in the tammar, other unidentified factors are presumably inherited in subpopulation(s) of cells of the unilaminar blastocyst. These could be either maternal determinants or transcriptional differences that are independent of the key lineage-specific transcription factors identified in the mouse.

Two distinct though not mutually exclusive models have been proposed for marsupial pluriblast–trophoblast segregation, which respectively share features with the inside–outside and cell polarity models proposed for eutherians. Both models depend on initial conceptus polarity. The first model proposes that as cell density is initially greater within the embryonic hemisphere during early cleavage, cell–cell contacts are greater there and could potentially provide positional information analogous to Hippo signaling in the mouse morula.[20,86] Thus, potential central–peripheral positional cues present during marsupial cleavage are analogous to the inside–outside positional cues operating in the eutherian morula. As the Hippo pathway is crucial to translating these positional cues in the mouse, Frankenberg et al.[86] hypothesized that it may also be active in specifying pluriblast precursors in marsupials. However, immunostaining of tammar conceptuses for YAP and WWTR1 demonstrated differential nuclear localization only after appearance of the embryonic disc, suggesting that Hippo signaling is not involved in trophoblast–pluriblast segregation in marsupials at least via the above proposed mechanism. Nevertheless, nuclear localization of WWTR1 (but not YAP) was specific to the trophoblast at least by the time of its morphological differentiation, suggesting a conserved role for trophoblast maintenance in mammals. The most common known roles for Hippo signaling are in regulation of organ size by density-dependent repression of proliferation, while there are comparatively few examples of roles for the pathway in lineage specification. During trophoblast–ICM differentiation in the mouse, outer cells do not proliferate at a significantly greater rate than inner cells. However, after appearance of the embryonic disc in the tammar, blastocyst expansion is mostly accounted for by expansion of the extraembryonic region. It is thus likely that the ancestral role of WWTR1 in the mammalian conceptus is to promote rapid proliferation of the trophoblast (Figure 6). This role would probably have also existed in mammalian ancestors with large yolky eggs, in which rapid proliferation of the extraembryonic ectoderm was required to fully envelope the yolk, as still occurs in monotremes. Differential activity of WWTR1 may have been later co-opted as a mechanism for early segregation of trophoblast and pluriblast lineages in eutherians by interacting with the core pluripotency gene regulatory network.

The second model for pluriblast–trophoblast segregation is an extension of the eutherian cell polarity model and aims to encompass all therian mammals.[3,8] The model proposes that totipotent polar cells, termed *polarblasts*, are essential precursors to pluriblast–trophoblast segregation. In the mouse morula, polarblasts are outer cells with stable

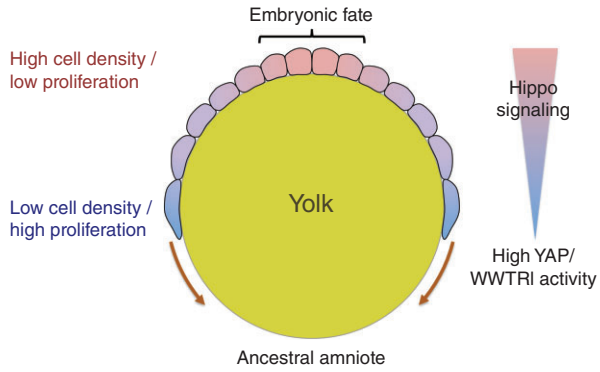

FIGURE 6 | Model for the evolution of a role for Hippo signaling in the mammalian trophoblast. In the large, yolky eggs of ancestral amniotes, rapid proliferation of extraembryonic ectoderm was necessary before the specification of embryonic cells.

apical–basal polarity that have the capacity to divide differentiatively to generate two different daughter cells: an apolar pluriblast (that does not inherit the apical domain) and another polarblast. Polarblasts that do not divide differentiatively can progress to differentiate into trophoblast. In translating this model to marsupials, the notion of a polarblast is best illustrated by the tier of blastomeres at the eight-cell stage of dasyurids. The fourth (8- to 16-cell) division is latitudinal, resulting in an upper tier of eight blastomeres located closer to the embryonic pole and a lower tier of slightly larger cells located closer to the abembryonic pole. The blastomeres of the latter tier inherit more vesicular material in their cytoplasm, which (possibly along with other maternal determinants) is initially asymmetrically distributed in the zygote. Whether such a mechanism for pluriblast specification is homologous between eutherians and marsupials is unclear because in marsupials apical–basal polarity develops perpendicular to the conceptus surface (where blastomeres adhere to the zona pellucida), whereas embryonic–abembryonic polarity is effectively tangential to this surface. However, it is possible that in both groups homologous determinants are involved, which in marsupials are maternally inherited and asymmetrically distributed in the zygote, while in eutherians they become associated with the apical domain of polarblasts. In the early unilaminar blastocysts of Afrotherians, all cells can be considered totipotent polarblasts, with a subset dividing differentiatively.

Hypoblast–Epiblast

Eutherians

The hypoblast is the second extraembryonic lineage to segregate during early mammalian development and gives rise to all extraembryonic endodermal components of the yolk sac. Aside from its role in the yolk sac, hypoblast derivatives are also sources of important signals to establish early axis polarity in the embryo proper (see Ref 87 for review).

Hypoblast specification in the mouse blastocyst was previously assumed to be dependent on positional cues acting on ICM cells adjacent to the blastocyst cavity, but more recent evidence showed that hypoblast and epiblast precursors are specified in an apparently stochastic manner in the earlier ICM.[88] In the early mouse blastocyst (32–64 cells), no spatial pattern of cell differentiation was observed by quantitative fluorescence and spatial data analysis of epiblast (NANOG) and hypoblast (GATA6) markers.[89] NANOG and GATA6 initially exhibit broad, overlapping expression in the morula and early blastocyst, but progressively become mutually exclusive within the ICM by around the 64-cell stage with a 'salt-and-pepper' distribution (Figure 5). Sorting of cells into epiblast and hypoblast layers is thought to occur via a process of possibly random cell movements, positional signals, and selective apoptosis.[90,91] A pattern of correlation between cell fate and position can be first observed within the mouse ICM at the 64- to 128-cell stage, when adjacent cells have a slightly higher chance of committing to the same lineage. This correlation is clear after the 128-cell stage, when hypoblast and epiblast are largely segregated.[89] This dynamic pattern of NANOG and GATA6 expression within the ICM, associated with specification of the epiblast and the hypoblast, seems to be conserved in other species like human and cow.[92,93] In the pig, however, NANOG could not be detected in the morula or early blastocysts.[71,94,95] NANOG can only be detected in the epiblast of the porcine blastocyst after 7.5 days of pregnancy, after the formation of the hypoblast, suggesting a different mechanism for epiblast and hypoblast specification in this species.[74,95]

Specification of hypoblast in the mouse depends on signaling by fibroblast growth factor (FGF)—an important signaling regulator involved in cell proliferation, differentiation, and migration. FGF4 is the most highly expressed FGF in the early embryo[96] and can activate downstream mitogen-activated protein kinase (MAPK) signaling through interaction with its associated receptor tyrosine kinases, FGFR1 and -2. In the mouse, the first indication that this pathway is critical for segregation of the epiblast and hypoblast came from characterizing the phenotype of mice mutant in the gene for growth factor receptor-bound protein 2 (GRB2), which activates the MAPK signal transduction pathway. Embryos lacking GRB2 formed blastocysts in which no cells expressing hypoblast markers were observed: the ICM was of normal size, but all cells expressed NANOG.[97] Chemical inhibition of the FGF/ERK pathway similarly results in an ICM of entirely epiblast identity.[98,99] Importantly, treating embryos with high levels of FGF4 resulted in the reciprocal phenotype where all ICM cells acquired hypoblast identity.[99] These data suggest that the segregation of the two lineages may be consolidated by reciprocal expression of FGFR in the hypoblast and FGF4 in the epiblast.[100] The same FGFR/MEK mechanism that restricts NANOG to epiblast cells has been recently implicated in restricting also the expression of SOX2 to these cells. In the epiblast, SOX2 then promotes expression of SOX17 in primitive endoderm cells by aiding in the

maintenance of the right level of FGF4.[82] Interestingly, the role of FGF/MAPK signaling in the constitution of the bovine ICM is not identical to that reported in the mouse. The ICM of bovine embryos treated with FGF4 is composed only of GATA6-positive cells. However, chemical inhibition of FGFR did not fully repress GATA6 expression. This data suggest that GATA6 expression in the bovine ICM may not be dependent on MAPK signaling, and the FGF4-induced expression of GATA6 is indirectly caused by the repressive effect of activated MAPK on NANOG.[92] In addition, MEK inhibition does not affect the NANOG-positive/GATA6-positive cell ratio in human blastocysts, also indicating that another signaling pathway may be involved in hypoblast specification in human.[92,93]

The establishment and maintenance of pluripotent cells in the ICM has also been studied by their *in vitro* counterpart, the culture of embryonic stem (ES) cells. Although FGF/ERK stimulation is the main trigger for ES cell differentiation,[101] its inhibition alone is not enough to support undifferentiated expansion of ES cells *in vitro*. It was reported that the inhibition of the glycogen synthase kinase-3 (GSK3) could improve self-renewal of ES cells.[102] The knowledge of the importance of these two signaling pathways on lineage specification of epiblast and hypoblast was applied to the elaboration of a protocol to derive ES cells in mice using their inhibitors, so-called *2i* conditions.[102] In addition, these conditions were sufficient for the establishment of ES cells from nonpermissive mouse strains such as C57Bl6, as well as from rat embryos, both of which were resistant to ES derivation under traditional culture conditions.[103,104] However, this approach has yielded limited benefit for the establishment of ES cell lines in other species, including the cow and human, where experimental data suggest that FGF/ERK signaling may be less important for lineage specification.[71] There have been several recent reports of deriving 'naïve' human ES cells, with properties more similar to mouse ES cells.[105,106] However, in all cases, a more complex mixture of growth factors and inhibitors was used than simply *2i* conditions. This suggests that we still have more to learn about the different pathways of lineage specification to the pluripotent epiblast in blastocysts and how to apply this knowledge to the derivation of true pluripotent stem cells *in vitro*.

Marsupials

Selwood[20] identified three main types of hypoblast formation in marsupials. In type 1, represented by *Didelphis* spp., some cells in one hemisphere of the very early, nonexpanded unilaminar blastocyst round up and move to the interior, where they form a transitorily multilayered aggregate of cells underlying the nascent epiblast. These hypoblast cells then continue to divide and spread along the inner surface of the blastocyst epithelium toward the abembryonic pole, eventually forming a complete bilaminar blastocyst. This represents a highly precocious mode of hypoblast formation in comparison to other marsupials. In types 2 and 3, hypoblast precursors appear only after a period of unilaminar blastocyst expansion. In type 2, the pluriblast is distinguishable from the trophoblast before hypoblast precursors become apparent. In type 3, all cells of the unilaminar blastocyst are morphologically indistinguishable until hypoblast precursors emerge. The distinction between types 2 and 3 may be trivial because morphological distinction of trophoblast from pluriblast is likely to depend on microscopy methods. Even in a single study on the dunnart, pluriblast and trophoblast were distinguishable only in some unilaminar blastocysts.[107]

A consensus among all authors who have examined marsupial hypoblast formation is that once hypoblast precursors are distinguishable, their fate is restricted. Thus, they separate completely from the blastocyst epithelium rather than remaining superficial and dividing asymmetrically to give rise to one hypoblast cell and one epiblast cell. This is consistent with the known mechanisms of epiblast–hypoblast segregation in the mouse. In a study using a *Pdgfra*^{GFP} reporter mouse line, hypoblast precursors marked by GFP expression were distributed heterogeneously throughout the ICM before sorting into an epithelium lining the blastocyst cavity.[91] Thus, delamination of hypoblast precursors in marsupials can be considered homologous to hypoblast–epiblast sorting in the mouse. In all marsupial species examined, hypoblast cells first emerge in small clumps near the periphery of the embryonic disc, suggesting that both stochastic mechanisms and signaling between the trophoblast and pluriblast play roles in hypoblast specification.

Only one study has examined marsupial hypoblast–epiblast differentiation at the molecular level.[86] In the tammar wallaby, the embryonic disc is only morphologically apparent once hypoblast precursors have already emerged (type 3). GATA6 was highly expressed in putative hypoblast precursors that had not yet delaminated, especially within more peripheral regions of the disc. By contrast, putative epiblast precursors expressed high levels of nuclear-localized NANOG and POU5F3. A notable difference with the mouse was that the total cytoplasmic

level of NANOG (as observed in mitotic cells) did not appear to differ between hypoblast and epiblast precursors, suggesting that at this early stage NANOG is regulated only at the level of nuclear localization and thus presumably downstream of GATA6. In monotremes, epiblast and hypoblast layers are also thought to arise by sorting of heterogeneously distributed hypoblast and epiblast precursors in the multilayered blastoderm, although this has not been examined at the molecular level.[108]

BLASTOCYST EXPANSION

Considerable diversity exists among mammals in the degree of blastocyst expansion. In some mammals, expansion continues until well after gastrulation when it is generally referred to as a chorionic vesicle, while in other species such as mouse and human, very little expansion occurs before implantation. In these species, implantation occurs early, only 4–4.5 days postfertilization (dpf) in the mouse, whereas the human blastocyst undergoes another round of cell divisions and implants 6–7 dpf.[109,110]

In all marsupial species, the blastocyst is surrounded by postovulatory coats formed by secretions from the reproductive tract.[15] A thick mucoid coat is deposited around the zona pellucida, followed by an outer, thin, shell coat. After completion of the blastocyst epithelium, a period of preliminary expansion occurs during which the mucoid layer is compressed between the stretching zona pellucida and the shell coat, which does not alter its dimensions. Once the mucoid coat is completely compressed, definitive expansion commences and the total volume enveloped by the shell increases. Although the shell stretches to accommodate this expansion, additional material continues to be added to it from uterine secretions.[111] The zona pellucida soon after thins until it is unidentifiable. The marsupial shell coat is thought to serve an important infrastructural role in supporting expansion of the blastocyst/chorionic vesicle as well as to provide an immunological barrier between fetal and maternal tissues. After around two-thirds of the gestation period, it breaks down to permit a superficial attachment (or invasive implantation in some species, especially bandicoots) of the choriovitelline membrane to the endometrium. By this stage amniogenesis is complete and thus a barrier between the embryo proper and maternal tissues is maintained.[112]

The ancestral eutherian is thought to have similarly possessed a choriovitelline placenta with a diffuse, epitheliochorial type of endometrial attachment,[113,114] thus requiring a degree of expansion to increase the surface area of the trophoblast prior to endometrial attachment. In all eutherians, the ICM/embryo proper is completely enveloped by the trophoblast at least initially, although in some species such as the rabbit, part of the trophoblast (Rauber's layer) regresses to leave the embryonic disc superficially located until after amnion formation. Notably such species possess additional postovulatory coats around the conceptus, although their homology with the marsupial coats is currently unclear.

In ungulate species, implantation is superficial and delayed. During this preattachment period, the large spherical horse conceptus migrates between the uterine horns many times a day, allowing interaction with the uterine luminal epithelium until day 18 of gestation,[115,116] about the same duration (day 18 of gestation) as in the tammar embryo.[117] Mucin-like glycoproteins secreted by the trophoblast during blastocyst formation are enclosed by the zona pellucida and form the blastocyst capsule shortly before zona loss.[118–120] The persistence of the blastocyst capsule prevents the elongation of the spherical horse embryo, allowing its mobility along the uterine lumen, and provides tensile strength to resist peristaltic myometrial contractions.[116,120] The pig and bovine embryos must undergo a drastic transformation from spherical to tubular and filamentous form that is achieved by growth and differentiation of the trophectoderm, resulting in an increased maternal–conceptus contact area.[121,122] The bovine conceptus grows more than 1000-fold during elongation from days 14 to 24 of gestation.[123] In the pig, elongation is a more dramatic process as the conceptus can reach more than 150 mm long within 4 h between days 11 and 12 of gestation.[121] The growth and differentiation of the trophoblast is necessary for antiluteolytic or luteotrophic protein secretion to maintain a functional corpus luteum, a phenomenon known as maternal recognition of the pregnancy. Similarly, there is a maternal recognition of pregnancy with the preattachment tammar embryo inducing and maintaining uterine secretory activity.[124,125]

DIAPAUSE

Many mammals can suspend their development for weeks or months at the blastocyst stage, a phenomenon known as embryonic diapause. There are around 94 eutherian species and 38 marsupial species that have diapause, but the morphological and molecular changes that take place during the onset and

reactivation from diapause have been examined in very few.[126,127] Diapause can be divided into three phases: arrest of cell division, maintenance of diapause, and reactivation after diapause. In most eutherians (except the roe deer), reactivation is closely coupled with implantation, but in marsupials placental attachment does not occur until much later, although changes in the uterus must still occur. Extracellular coats separate the blastocyst from the endometrium during diapause in all species except rodents, in which zona pellucida dissolution occurs prior to dormancy. In carnivores, the zona persists during diapause.[128]

Maternal Control of Diapause

During diapause, the tammar blastocyst is maintained in a metabolically inactive state during which cell division is entirely inhibited,[126,129] while in the mouse there is a very small increase in cell number.[130] Maintenance of pluripotency in the mouse epiblast during diapause depends on leukemia inhibitory factor (LIF), which is expressed by the trophoblast. It has been proposed that the role of LIF in maintaining epiblast pluripotency specifically during diapause provides a rationale for the amenability of mice to derivation of ESCs.[130,131] Thus, the naïve state of pluripotency described in mouse ESCs could relate to the presence of diapause in this species.[132] Diapause is initiated in the trophoblast prior to the ICM,[133] but proliferation after reactivation begins in the ICM before the trophoblast.[134] Blastocyst reactivation triggers a phenotypic transformation that is essential for it to be able to implant, including a transition of the trophoblast from an epithelial to invasive morphology.[135]

Diapause can be either obligatory (e.g., controlled by photoperiod) or facultative (e.g., controlled by lactation or nutrition). The precise cellular mechanisms that suspend development of the blastocyst are not well understood, but there are some interesting new data from mouse, mink, and tammar. Its control is best understood in rodents, especially the mouse in which diapause is induced by lactation. In the mink, diapause is under seasonal (photoperiod) control while in marsupials, especially the tammar, both lactation and photoperiod can induce diapause.[9,126,136–145] Interestingly, there is only one ungulate with diapause, the roe deer, and it was the first species in which diapause was recognized. Bats are a special case and several different forms of delay occur in the chiropterids: delayed fertilization, delayed development, and embryonic diapause.[9,146,147] There have been a number of reviews summarizing the seasonal and lactational control of diapause, and the environmental, physiological, and metabolic signals that control it, but our knowledge of the molecular controls at the uterine-blastocyst interface is just beginning to emerge. Distal mechanisms controlling diapause will be discussed only briefly here, with more attention focused on the proximal interactions between the endometrium and the blastocyst (Figure 7).

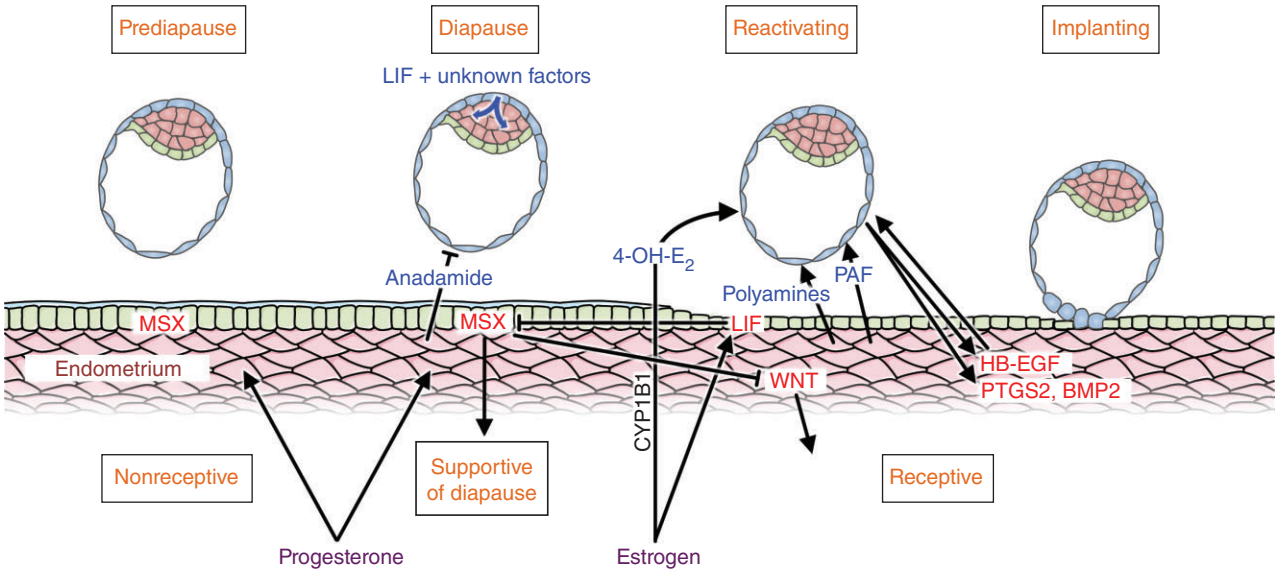

FIGURE 7 | Mechanisms regulating diapause in the mouse. There is evidence for conserved roles for many of these factors in both tammar and mink, including progesterone, *MSX* genes, leukemia inhibitory factor, polyamines, and platelet-activation factor.

In most species, the ovary participates in the control of diapause with the exception of the roe deer in which it appears not to be involved. In the tammar, prolactin released from the pituitary maintains lactational diapause by inhibiting the corpus luteum; thus, suppression of prolactin results in blastocyst reactivation. During seasonal diapause, photoperiod and the pineal hormone melatonin maintain seasonal diapause. By contrast in the mink, prolactin stimulates the corpus luteum to induce reactivation. During reactivation in both species, the corpus luteum stimulates the uterus by releasing progesterone (and other unknown factor(s) in the mink). In the mouse, reactivation requires progesterone as well as a surge of estrogen released from the ovary. Estradiol injection does not successfully induce reactivation in the mink[148] or in the tammar.[149]

Uterine Factors Regulating Diapause

In the mouse, progesterone and estradiol induce endometrial proliferation and the production and release of some cytokines and growth factors from the uterus that can have both autocrine and paracrine actions to regulate the preimplantation conceptus and prepare the endometrium for implantation.[150] These appear to have an important role in stimulating subsequent resumption of embryonic growth after embryonic diapause.[126] One of the main effects of the nidatory estrogen pulse is to stimulate the endometrial glands to secrete LIF into the uterine lumen where it acts on the luminal epithelium. LIF is essential for preparing the uterus for receptivity to implantation; in its absence, the uterus remains nonreceptive and blastocysts enter diapause. This role for uterine LIF is apparently unrelated to the role of trophoblast-secreted LIF in maintaining the epiblast.[130] One of the key effects of LIF is to downregulate *Msx* genes (*Msx 1* and *Msx 2*), which encode homeobox transcription factors essential for preparation of the uterus prior to implantation.[151–154] *Msx* genes appear to have two distinct roles: the first is to halt proliferation of the luminal epithelium and progress it along a path toward differentiation that is essential for implantation; the second is to inhibit the final process of differentiation. Concordantly, *Msx* genes are expressed in the endometrium throughout preimplantation development and abruptly downregulated at implantation. One of the major roles of *Msx* genes is to negatively regulate WNT ligands, which promote proliferation of the luminal epithelium and stroma. In the case of diapause, *Msx* genes are additionally expressed during the dormant phase and downregulated at the time of blastocyst reactivation. During diapause in mice with both *Msx1* and *Msx2* conditionally deleted in the uterus, blastocysts do not receive the appropriate signals to enter diapause but are also unable to implant as the endometrium is nonreceptive. However, they can still stimulate localized upregulation of early implantation markers such as *Ptgs2* (*Cox2*) and *Bmp2* in the endometrium.[151] Based on expression analysis in both mink and tammar, the role of *MSX* genes in early pregnancy and diapause appears to be evolutionarily conserved.[151]

A number of factors have been implicated in the cross-talk between the blastocyst and the uterus that regulate both implantation and diapause. Evidence exists both for factors that actively induce blastocyst quiescence and for factors that stimulate its reactivation. Although mouse blastocysts can reactivate their metabolism *in vitro*, they are not fully competent for implantation.[155–166] The cannabinoid anandamide potentially acts as a negative regulator as it is downregulated in the mouse uterus during reactivation *in vivo*, whereas at elevated levels *in vitro* it inhibits blastocyst reactivation.[167]

Several factors have been implicated in positively stimulating the blastocyst during reactivation. The nidatory estrogen surge stimulates preparation of the endometrium, where the estrogen (estradiol-17β) is also converted by the enzyme CYP1B1 to the catecholestrogen 4-hydroxyestradiol (4-OH-E2). This estradiol metabolite is required for blastocyst reactivation *in vitro*,[168] while CYP1B1 is expressed in the uterus close to sites of implantation.[169] Another factor, PAF (originally named as platelet-activation factor), a phospholipid present in the endometrium, interacts with PAF receptor (PTAFR), which is expressed in the embryos of mouse, rabbit, hamster, and human.[170–174] PAF appears to stimulate embryonic metabolism, cell proliferation, and viability, and may be important for maternal recognition of pregnancy.[173] In the tammar during diapause, PAF is low but endometrial PAF increases *in vitro* around the time of reactivation.[175,176] While there is some evidence to suggest that endometrial PAF is involved in reactivation, it is as yet uncertain whether it is essential.

Several other factors are also upregulated specifically at the early implantation site in the mouse, including BMP2, PTGS2 (COX-2), and HB-EGF.[136] HB-EGF is a strong candidate for signaling to the blastocyst to stimulate reactivation. It is expressed in the uterus in response to nidatory estrogen, but also becomes enriched at localized sites close to blastocysts several hours before attachment, even before zona pellucida dissolution during gestation without

diapause.[136,177] The blastocyst also upregulates both HB-EGF and its receptors ERBB1 and ERBB4 during reactivation, suggesting that this cross-talk is crucial in preparing the blastocyst for implantation.[178,179]

Polyamines (putrescine, spermidine, and spermine) represent an additional class of factors that have been implicated in direct signaling to the blastocyst to control diapause. Polyamines regulate cell cycling and protein synthesis, and uterine genes involved in polyamine metabolism appear to be important for embryo implantation.[180–182] Polyamine synthesis is rate-limited by the ornithine decarboxylase-1 (*ODC1*) gene.[183] In the mouse during diapause, ODC1 and putrescine are upregulated at reactivation[184] and in the mink are upregulated at the termination of diapause.[138,140,185,186] The mechanisms of action of the polyamines in diapause are not yet defined, but presumably they act by inhibiting cell proliferation and arrest of the cell cycle. Treating mink with an ornithine decarboxylase inhibitor, which reduces polyamine levels in the uterus, is able to rearrest cell proliferation in newly reactivated mink blastocysts.[185] The duration of ODC1 inhibitor-induced diapause depends on the duration of treatment, and if withdrawn, the embryos resumed development. Similarly, administration of this inhibitor on the day of implantation arrests embryo development in the mouse, rat, and hamster.[187–190] Collectively, this is strong evidence that polyamines are critical factors in the reactivation of diapausing blastocysts. It will be of interest to determine the role of polyamines in species other than rodents and mink.

Evolution of Diapause

Many other factors have been identified from large-scale transcriptomic or proteomic screens as candidate regulators of diapause.[178,191–193] Despite the many different molecular controls of diapause, there appears to be a surprising level of conservation of the factors involved, suggesting an ancient evolutionary origin. There is recent speculation that diapause is an ancestral condition in mammals.[194] Blastocyst transfer across species during diapause was an obvious test of its universality, yet has been done very few times. In the marsupial quokka (*Setonix brachyurus*) diapausing blastocysts can be successfully reactivated after transfer to reactivated uteri[195] and also in the tammar.[196] Ferrets do not have embryonic diapause, but reciprocal transfer of mink and ferret blastocysts to diapausing mink uteri induces diapause in ferret blastocysts, whilst the converse results in activation of mink blastocysts.[197] Attempts at similar reciprocal transfers between brushtail possum and tammar were not successful (CH Tyndale-Biscoe and MB Renfree, unpublished results), but mouse blastocysts increase uridine uptake when incubated in reactivated tammar uterine secretions[198] and glucose uptake and lactate production.[157] Most recently, reversible embryonic diapause can be induced in sheep blastocysts after embryo transfer to the uteri of mice in diapause, resulting in a period of quiescence of the sheep blastocyst until reimplantation into sheep uteri. These data suggest that the potential for diapause is not restricted to the species where it is known to occur and may indeed be the ancestral condition.[194] Taking up an early suggestion that human conceptuses may be able to enter diapause, Ptak, Tarin and coworkers[199,200] concur that diapause may even occur in humans. Although embryonic diapause could be a universal characteristic of mammalian blastocysts, much more evidence is needed and more details on the molecular control of the 120 species with confirmed embryonic arrest at the blastocyst stage.

SUMMARY AND CONCLUSIONS

The mammalian blastocyst evolved in concert with the placenta and an associated reduction in conceptus volume due to a progressive loss of yolk. Although serving a common purpose, the blastocyst shows remarkable variation between species, both morphologically and in the molecular mechanisms regulating early lineage specification. This evolutionary plasticity can also be considered to have driven the significant diversity in modes of placentation found in mammals. The assignment of a meaningful, universal definition of the blastocyst stage is complicated by variation in the degree of expansion before placental attachment or implantation and in the timing of early differentiation events. Nevertheless, it may be recognized that the blastocyst was a necessary adaptation for the progressive loss of yolk that occurred during the evolution of viviparity in mammals. Blastocyst expansion compensates for reduced ovum size and allows mammals to conform to the typical amniote mode of development. The extracellular coats that envelope the blastocysts of many species are also remarkably diverse and much remains to be learned of their role in interactions between the conceptus and the uterine environment. There is a significant need for more research on the blastocysts of lesser known mammals, including monotremes, marsupials, and Xenarthrans, to gain a proper understanding of the evolution of early mammalian

development. Technologies such as transcription activator-like effector nucleases (TALENs), zinc-finger nucleases (ZFNs), and especially the RNA-guided CRISPR-Cas9 system have recently allowed the precise editing of endogenous genomic loci. Together with whole genome sequencing, these tools offer researchers new possibilities to investigate the molecular mechanisms involved in early lineage segregation in unconventional laboratory species, once restricted to the mouse due to its amenability to ESC derivation and manipulation. The blastocyst is also the stage at which many species undergo embryonic diapause; a full understanding of the molecular mechanisms regulating this phenomenon is likely to have far-reaching benefits for basic biology of the cell cycle and particularly cancer.

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