REGULATION OF BLASTOCYST FORMATION

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1. ABSTRACT

Preimplantation or pre-attachment development encompasses the "free"-living period of mammalian embryogenesis, which directs development of the zygote through to the blastocyst stage. Blastocyst formation is essential for implantation, establishment of pregnancy and is a principal determinant of embryo quality prior to embryo transfer. Cavitation (blastocyst formation) is driven by the expression of specific sets of gene products that direct the acquisition of cell polarity within the trophectoderm, which is both the first epithelium of development and the outer cell layer encircling the inner cell mass of the blastocyst. Critical gene families controlling these events include: the E-cadherin-catenin cell adhesion family, the tight junction gene family, the Na/K-ATPase gene family and perhaps the aquaporin gene family. This review will update the roles of each of these gene families in trophectoderm differentiation and blastocyst formation. The current principal hypothesis under investigation is that blastocyst formation is mediated by a trans-trophectoderm ion gradient(s) established, in part, by Na/K-ATPase, which drives the movement of water through aquaporins (AQPs) across the epithelium into the extracellular space of the blastocyst to form the fluid-filled blastocoel. The trophectoderm tight junctional permeability seal regulates the leakage of blastocoel fluid, and also assists in the maintenance of a polarized Na/K-ATPase distribution to the basolateral plasma membrane domain of the mural trophectoderm. The cell-to-cell adhesion provided by the E-cadherin-catenin gene families is required for the establishment of the tight junction seal and the maintenance of the polarized Na/K-ATPase distribution. Blastocyst formation is therefore directly linked with trophectoderm cell differentiation, which arises through fundamental cell biological processes that are associated with the establishment of cell polarity.

2. INTRODUCTION

"This chapter is dedicated to the spirit of Lynn Wiley whose research accomplishments provided great insight into the regulation of blastocyst formation and inspired others to pursue this exciting research area as well"

The first week of development represents a perilous time for the early conceptus as the majority of inseminated oocytes fail to successfully complete this developmental interval to implant to the uterine wall and establish a pregnancy (1-5). This interval called preimplantation or pre-attachment development (depending upon the species) is uniquely mammalian and encompasses the free-living period of mammalian development during which the early conceptus traverses the oviduct and gains access to the uterine environment (1-6). This period culminates in the formation of an embryonic structure called the blastocyst, which is composed of the outer epithelial trophectoderm, a fluid-filled cavity and small group of cells called the inner cell mass, which are the

progenitors of the embryo proper (6-12). The blastocyst is not simply the vessel that delivers its cargo of inner cell mass cells to the uterus as it contains the specialized cell type (the trophectoderm), which will mediate the maternal-fetal interactions required to support eutherian embryogenesis. The trophectoderm cells differentiate into the trophoblast to mediate either invasion or attachment to the uterus during implantation and as a prelude to their subsequent contribution to placental structures (6-12). Blastocyst formation is therefore essential for the establishment of pregnancy and is intimately coordinated by the events controlling trophectoderm differentiation.

Decades of research have elucidated many of the cell biological and molecular mechanisms that control this developmental interval. Although the mouse embryo has been the animal model of choice for most of the experimentation, the extension of these studies to the human and domestic agricultural species has stimulated vigorous research activity in these embryo systems leading to explosive growth in the fields of assisted reproductive technology and embryo transfer in humans and other species (1-12). Despite these advances, pregnancy rates following embryo transfer remain lower than expectations for all species, and further advances are required to optimize the general application of these technologies (1-6). Areas of research include; ovarian stimulation; folliculogenesis and oocyte maturation; embryo culture and metabolism; hormonal and growth factor regulation; gamete micromanipulation (intracytoplasmic sperm injection; ooplasmic injection); genomics and gene expression; uterine receptivity and synchronization; embryonic signaling of maternal recognition of pregnancy; organ, gamete and embryo cyropreservation and embryo cloning, transgenesis, and gene targeting in animal species.

The purpose of this review is to outline the critical events in the early developmental program that regulate blastocyst formation. It is hoped that by reviewing this important research area, specific avenues for further research will be revealed supporting a greater understanding of the mechanisms controlling preimplantation or pre-attachment development. This information is required to promote the safe and efficient production of normal mammalian embryos and offspring in assisted reproductive technology and embryo transfer applications.

3. PREIMPLANTATION DEVELOPMENT

Development begins during gametogenesis within the ovaries and testes of the parental animals. The spermatozoon contributes one set of paternal chromosomes to the embryo at the time of fertilization. The maternal chromosomes of the ovulated oocyte arrest at metaphase II following ovulation to await insemination by a fertilizing spermatozoa. Following fertilization, the zygote undergoes several cleavage divisions that divide the ooplasm into smaller compartments or blastomeres. The first morphogenetic event, compaction, arises following the third cleavage division in the mouse, although the timing of this event varies greatly among the mammalian species

arising, for example, at the 32-64 cell embryo stage in the bovine embryo (4, 13-17). Compaction represents the onset of cellular differentiation during mammalian development as the outer cells of the conceptus begin to polarize as a prelude to trophectoderm differentiation (7, 17, 18). Blastocyst formation is the morphogenetic event that follows compaction in this continuum of preimplantation development (1-12). Each succeeding phase of this developmental interval is dependent upon preceding events. It is therefore not possible to consider the mechanisms controlling blastocyst formation by focusing exclusively on the blastocyst. We will begin by briefly examining the oogenetic to embryonic transition and compaction.

3.1. Oogenetic to Embryonic Transition

Mammalian development is initially sustained by gene transcripts and polypeptides produced and stored in the oocyte during oogenesis (19). After one to several cleavage divisions (depending upon the species), genetic control of development comes predominantly under the embryo's control as oogenetic gene products decline, and embryonic transcription begins (19-29). The transition from oogenetic to embryonic control of development or maternal zygotic transition (MZT) varies in its timing between species (4, 22, 30). This is a critical event as blastocyst formation is dependent on expression of embryonic genes (31-33). However, it must also be kept in mind that specific oogenetic products (for example E-cadherin) can influence development to at least the blastocyst stage and potentially later in development. Since this topic is the subject of several reviews (for example 19, 22, 30, 34, 35) including a chapter in this issue only the principal components will be briefly summarized. Mouse embryos cultured from the one-cell stage in the presence of transcriptional inhibitors do not develop beyond the two-cell stage, indicating that new transcription from the zygote genome is, necessary for development beyond this point (19-29). During this transition, the majority of maternal mRNA molecules accumulated during oogenesis are degraded and replaced by new mRNA molecules (20-24, 28, 36). These fluctuations in mRNA populations parallel marked changes in protein synthetic patterns between the 1- to 4-cell stages (27, 33, 35, 37). Similar observations have been obtained from studies applied to other species however the timing may be delayed compared to the mouse (38,39). For example, (^{3}H)-uridine incorporation was first detected at the end of the bovine eight-cell stage (38, 39). However, lower levels of (^{3}H)-uridine incorporation have been reported in two- and four-cell bovine embryos suggesting that a minor degree of transcriptional activity precedes the larger full activation of the embryonic genome (37, 38, 40, 41). This may in fact be a characteristic of all species. Bovine embryos cultured from the one- to four cell stages in the presence of alpha-amanitin, progress to the four/eight-cell stage but none develop to the 16-cell stage (42, 43).

Many of the transcripts expressed at the 2-cell stage of mouse development continue to accumulate as development progresses to the blastocyst stage. However, there are a number of additional transcripts important for cell proliferation, compaction, and blastocoel formation
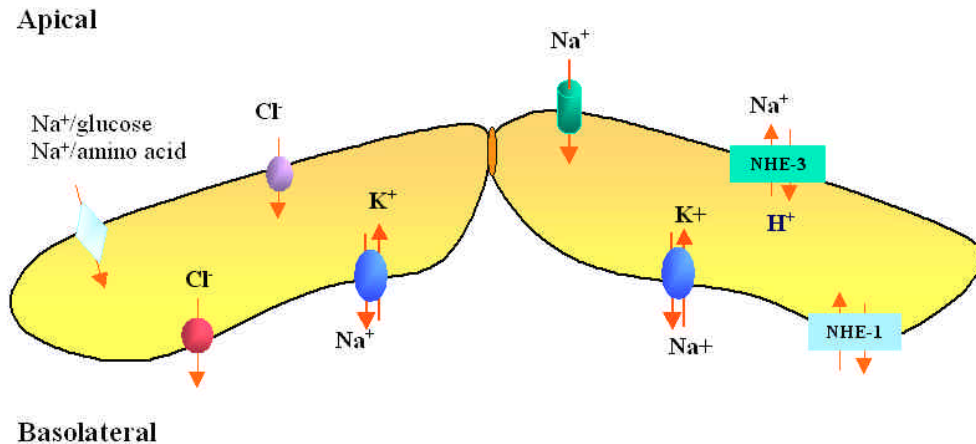
Figure 1. Expression of ion transporters and enzymes in the trophectoderm of mammalian preimplantation embryos.

(cavitation) that are not expressed until after the 2-cell stage (33, 35). The morphological changes in embryos that are necessary for blastocyst formation are in fact tightly coupled to ongoing transcriptional events (26). The variation in the patterns of newly synthesized polypeptides observed on two-dimensional gels between the 4-cell to blastocyst stage are not as marked as those between the 1- to 4-cell embryo stages, but several new gene products undergo a transcriptionally-dependent increase during this interval (44).

3.2. Compaction

Compaction is a common feature of preimplantation development within all eutherian mammalian embryos, although the timing of this event varies greatly arising later in the pre-attachment embryos of the agricultural domestic species (4). Compacting embryos undergo an increase in interblastomeric contact that obscures the distinct individual cell boundaries and continues until the embryo ultimately appears as a uniform cell mass called a morula (7, 14-17). The cellular events associated with compaction include: (i) the development of Ca^{2+} dependent cell adhesion; (ii) the establishment of gap junction mediated interblastomeric cell communication; (iii) the initiation of cell contact-induced cell polarization, and (iv) the appearance of focal tight junctions which eventually divide the plasma membrane of the outer blastomeres into apical and basolateral membrane domains (15-17). These events, particularly the development of asymmetrical cell contact and polarity within the outer blastomeres are essential and directly contribute to the formation of the blastocyst. By the late morula stage, the embryo acquires the remaining macromolecules that are necessary for blastocyst formation (cavitation) to occur (31-33).

3.3. Blastocyst Formation (Cavitation)

The trophectoderm acquires the capacity to initiate and regulate the events of cavitation by expressing gene products that facilitate the transport and retention of the blastocoelic fluid as it accumulates in the nascent blastocyst cavity. Therefore, for cavitation to arise, the embryo relies upon the development of a polarized epithelium (6-8, 11, 12). The blastocoelic fluid is predominantly water, thus the mechanism of its production and accumulation relies on the ion transport properties of the trophectoderm (12, 45-48). In many ways, the trophectoderm is comparable to an early kidney tubule epithelium as many ion and water transport characteristics are shared between these epithelia. The trophectoderm is an ideal model for epithelial cell differentiation as this epithelium arises "de novo" from apolar blastomeres. For these reasons blastocyst formation is tightly coupled to the development of trophectoderm ion transport systems, the epithelial junctional complex, and the primary role played by establishment of cell-to-cell adhesion between blastomeres during compaction (6-8, 11, 12; see Figure 1).

The basis for these conclusions stemmed initially from isotope flux measurements and electron probe microanalysis of Na^+, Cl^-, K^+, Ca^{2+}, and Mg^{2+} in the murine blastocyst fluid (49-55). These studies demonstrated that all of these ions were concentrated within the blastocoelic fluid (49-55). Active transport would be required to move these ions against their concentration gradients. The transport of amino acids such as glycine and alanine is also linked to a sodium-dependent mechanism that develops in the blastocyst (54-58). The vectorial transport of Na^+ and Cl^- (but not K^+) from the medium is essential for the onset and progression of cavitation (52). The Na^+ ions likely enter the trophectoderm cells through apically localized Na^+ channels and/or via various Na^+ co-transporters such as the Na^+/H^+ exchanger (52) or Na^+/glucose co-transport system (59). The Cl^- transport was initially thought to be paracellular, and thus regulated by the epithelial junctional complex (52). More recent studies have determined that Cl- transport is also mediated across the epithelium (47, 48, 60). It has been proposed that the trans-trophectoderm Na^+ gradient is completed by the active transport of Na^+ out of the cell into the blastocoelic cavity by a basolaterally localized Na/K-ATPase (9, 60, 62). The presence of such a gradient would result in the movement of water down the gradient across the epithelium and into the blastocyst cavity (6, 8, 11, 62). A great deal of evidence collected in the past

twenty years supports this hypothesis as it has been determined that the Na/K-ATPase is present in the basolateral plasma membrane domain of the trophectoderm for several species including the mouse, rabbit, pig, and cow (6-12, 31, 46, 52, 55, 61-75).

Cavitation is thus a complex cellular process that requires the precise coordination of several cellular events. Na/K-ATPase distribution is influenced by the stability of the trophectoderm tight junctional seal (76-84); the membrane cytoskeleton (79) and by E-cadherin mediated cell adhesion (67, 85-87). We will now turn our attention to the establishment of cell polarity in the outer embryonic blastomeres as this event not only drives trophectoderm cell differentiation but also is the foundation of blastocyst formation.

4. TROPHECTODERM DIFFERENTIATION

4.1. Cell Polarity and the Inside Outside Hypothesis

Cell polarity in the preimplantation embryo is characterized by the establishment of; (1) cell surface components such as apical microvilli and associated lectin receptors (reviewed by (88), (2) asymmetrical distributed of membrane proteins between the apical and basolateral plasma membrane domains (6, 12), and (3) specific cytoplasmic components (ie. the apical aggregation of actin bundles and endocytic vesicles; (88)) and the basal migration of mitochondria and lipid vesicles (9). As interblastomeric cell-to-cell contact increases during compaction, the formation of "free" and "apposed" plasma membrane regions occurs and by the 16-cell stage, the mouse embryo is composed of an outer layer of polar cells that completely encloses an inner group of 4-7 apolar blastomeres. The degree of cell contact associated with cell position provides the necessary developmental cue for the maintenance of polarity within the outer blastomeres (for review see (17). The "free" or apical membrane surfaces of the outer blastomeres develop a cytochalasin-D resistant microvillus cap (80), Na^+-dependent amino acid transport systems (57), a Na^+/glucose co-transporter (59) and Na^+-channels (52). The basolateral surfaces of the outer blastomeres remain free of microvilli but become distinguished from the apical surfaces by the localization of the tight junction ZO-1 polypeptide (84), gap junctions (87), and E-cadherin (uvomorulin; (90)). The polarity exhibited by the outer blastomeres is displayed within the cell cortex by the appearance of an apical actin cap and within the cytoplasm by the asymmetric distribution of lipid vesicles, mitochondria and the nucleus towards the basal poles of the cells (8, 91). This cell polarity is very stable, as it is maintained even in the presence of inhibitors (such as cytochalasin B or D) that block the cell flattening, the development of cell adhesion, and the establishment of cell coupling via gap junctions (16). Although asymmetrical cell contact is the principal spatial cue in the development of trophectodermal cell polarity (reviewed by (88)), directional ion currents have also been implicated. Nuccitelli and Wiley (92) showed that individual polarized morula stage blastomeres exhibit a positive current flowing through their apical end and out their basal end. The ion current polarization hypothesis suggests that an asymmetry in inward current between the free and apposed cell surfaces could facilitate cell polarization (10, 92). This current may interact with the cytoskeleton via the plasma membrane to polarize the cellular positions of the nucleus and mitochondria to the basal cell ends (8).

4.2. Cell-to Cell adhesion

E-cadherin (uvomorulin) is the principal molecular component of the adherent junction. Stable cell-to-cell contacts and adhesion plaques arise through associations of E-cadherin with the actin cytoskeleton mediated by interactions with alpha-catenin, beta-catenin and gamma-catenin (93-96). The pivotal role played by E-cadherin during epithelial differentiation has been demonstrated by transfection of non-epithelial cell lines with cadherins to induce a polarized epithelial phenotype (93). Cells transfected with the brain-associated cadherin, B-cadherin, do not polarize (93). E-cadherin is at the top of the cascade of protein interactions that drive trophectoderm cell differentiation. E-cadherin, and catenins, as well as cytoskeletal actin are expressed and stored in the oocyte during oogenesis; therefore, they are present in the early mammalian embryo from fertilization onward (97, 98). However, stable E-cadherin mediated cell-to-cell adhesion does not arise until compaction is initiated (87, 99, 100). The factors or mechanisms that control the suppression of stable cell-to-cell adhesion in the early embryo prior to compaction represent a new area of research that requires further attention.

The dependence of compaction and cavitation upon E-cadherin mediated events has been demonstrated by treating pre-compaction murine embryos with E-cadherin blocking antisera (64, 67, 85). Embryos treated with an antibody to E-cadherin do not undergo compaction and do not proceed with a normal cavitation (67). Under these conditions, the epithelial junctional complex does not form. It appears that E-cadherin, possibly working in concert with the membrane cytoskeleton, contributes to the establishment of the epithelial junctional complex and the insertion of Na/K-ATPase into the basolateral membranes. More recently, gene-targeting approaches by homologous recombination methods have been employed to produce E-cadherin null mutants (101,102). E-cadherin null embryos undergo compaction (an event contributed to residual oogenetic E-cadherin proteins) but fail to develop into normal blastocysts and never hatch from the zona pellucida (101, 102). Removal of E-cadherin does not prevent cell polarization but rather delays and randomizes the orientation of cell polarity preventing the formation of an ordered trophectoderm cell layer (101,102). Further analysis of these null mutant embryos revealed that both alpha- and beta-catenin are down regulated and that ZO-1 expression is not detectable (103). These studies clearly demonstrate that E-cadherin plays a pivotal role in differentiation of the trophectoderm, and thus has a central role in supporting further embryonic development (see figure 2).

Similarly, the trophectoderm epithelium of alpha-catenin null mutants is abnormal, and development is halted at the early blastocyst stage (97). In contrast, murine
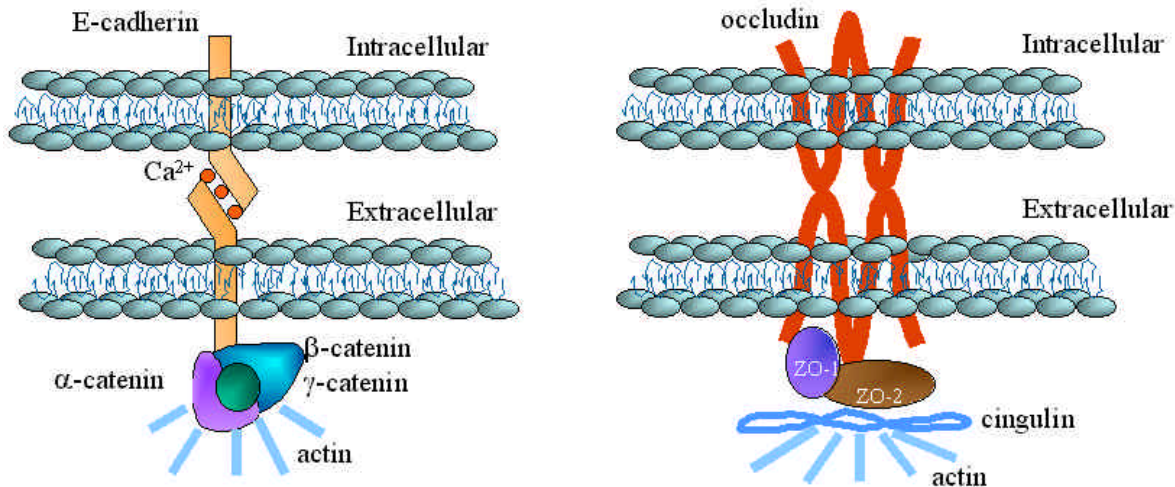
Figure 2. Molecular organization of the zonula adherens (left side) and zonula occludens (right side) in polarized epithelial cells.

beta-catenin null mutants undergo cavitation and continue to develop until gastrulation (100). Maternal (oogenetic) stores of beta-catenin may suffice during these early events and/or gamma-catenin, may also bind to the cytoplasmic domain of E-cadherin, mediating interactions with alpha-catenin (104-106). It follows that two distinct E-cadherin/catenin complexes occur within the same cell, specifically, E-cadherin/beta-catenin and E-cadherin/gamma-catenin (81). Gamma-catenin, is the only known protein shared by desmosomes and adherens junctions (AJs) (81). Development of gamma-catenin null mutants generally ceases at day 10.5 of gestation (101, 105). Embryonic gamma-catenin is synthesized from the murine 8-cell stage onward, but the protein does not become localized to cell membranes until the morula stage (following compaction), where it is present in all membrane contact sites, including those of the inner cell mass (81). Thus, although all components of the AJ are implicated in compaction and trophectoderm differentiation, their roles are variable.

Adherens junctions (AJs) tightly regulated, yet dynamic structures, given that movement requires constant disruption of some cell contacts with formation of new ones, without overall loss of adhesive strength. The presence of AJ components in a developing oocyte suggests that a secondary programming step is required to either trigger or block inhibition of their association. A viable hypothesis for the delay of AJ formation in the early mouse embryo is that an inhibitor or repressor of E-cadherin/catenin interactions represses the establishment of stable adhesion complexes. Recent studies have revealed a potential role for IQGAPs, targets of the Rho GTPases (Cdc42 and Rac1) in inhibition of E-cadherin/catenin interactions (107-111). IQGAP1 binds to the amino terminus of beta-catenin complexed with E-cadherin *in vivo* and *in vitro*, promoting dissociation of alpha-catenin, thereby disrupting cadherin-mediated cell adhesion (108-111).

The Rho GTPases are 20-30 kDa GTP-binding proteins that belong to the Ras superfamily. Rho GTPases include Rho A, B, C, D, and E, Rac 1 and 2, RacE, Cdc42Hs and TC10 (112-115). RhoA, Cdc42 and Rac1 have been most extensively studied. These three GTPases regulate membrane ruffling, cell motility, actin polymerization and cell-cell adhesion (112-115). Rho activity is modulated in a cyclical fashion. Effectively, these small GTPases act as molecular switches, alternating between active GTP-bound and inactive GDP-bound states. The latter may be promoted by GTPase activating proteins (GAPs), such as Rho GAP, which down-regulates GTPase activity. Recent studies in keratinocytes and other epithelial cells have determined that Rho GTPases are also involved in the regulation of cadherin mediated cell-cell adhesion (109, 110, 113, 116, 117). This conclusion is further supported by reports that demonstrate that over expression of dominant-negative Cdc42 and/or Rac1 reduces cadherin-mediated cell-cell adhesion (109, 110).

A number of Cdc42 and Rac1 effector proteins have been identified. Of these, only IQGAPs are implicated in the regulation of cell adhesion. Two IQGAP forms (1 and 2) are known. These large (>180 *kD*) proteins appear to serve as scaffolds, and contain several specific protein interacting domains, including a calponin-related F-actin binding segment, a WW domain, four calmodulin IQ motifs, and 5-6 IQGAP repeats, the function of which has not been determined (112, 113-116). They lack an arginine finger residue in their Ras GAP domain, and may not have significant GAP activity (109, 110). Both IQGAPs interact with Cdc42 and Rac1 to inhibit their GTPase activity; thus maintaining them in an active, GTP-bound state (108-111, 116-122). The interaction of Cdc42 or Rac1 with IQGAP1 inhibits it from binding to beta-catenin. This allows alpha-catenin to associate with beta-catenin, stabilizing cadherin-mediated adhesion (109, 110). Although these interactions of cadherins/catenins/Rho GTPases and IQGAPs have not as yet been explored during the first week of development
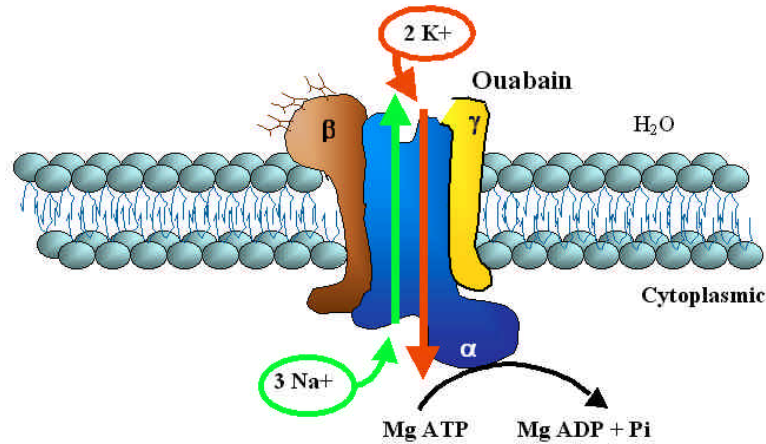
Figure 3. Structure and characteristics of the Na/K-ATPase.

we would predict that this area of research would be fruitful for revealing the mechanisms that trigger the onset of stable cell-to-cell adhesion and thus the onset of compaction and cell polarity in outer blastomeres during preimplantation development.

4.3. Establishment of Trophectoderm Tight Junctions

The tight junction (or zonula occludens) consists of a complex of several proteins including ZO-1, ZO-2, 7H6, cingulin, occludin and claudins (123; see figure 2). The zonula occludens appears as a series of close membrane contacts or "kisses" (84). In freeze-fracture the junction forms a series of anatomizing fibrils (reviewed by (124, 125)). Occludin and the claudins are the core integral membrane proteins that interact with ZO-1 (a 220 kDa peripheral membrane protein) and cingulin to form a link between the tight junction and the cytoskeleton (123-129). The tight junction serves at least two functions, these being the regulation of paracellular transport (the movement of water and solutes between epithelial cells) and maintenance of epithelial cell polarity. These roles are first revealed during trophectoderm cell differentiation and blastocyst formation (130, 131). The development of the junctional complex is initiated within the 8-cell embryo but requires an additional two cleavage divisions to become complete in the blastocyst (76-78, 84). The impermeable seal does not form until after cavitation has begun and it is only after the formation of this seal that the leakage of blastocoelic fluid is regulated, allowing the embryo to expand in size due to fluid accumulation (77). The epithelial junctional complex, along with the membrane cytoskeleton, almost certainly contributes to the maintenance of the polarized Na/K-ATPase distribution within the trophectoderm (67). If the junctional complex and the cortical actin cytoskeleton are disrupted by the incubation of mouse blastocysts in medium containing cytochalasin B or D, the Na/K-ATPase polarized basolateral distribution is also disrupted and the enzyme assumes an apolar distribution encircling the outer margins of the trophectoderm cells (67). These embryos can recover and proceed normally through cavitation if they are subsequently cultured in medium without cytochalasin. This experiment demonstrates that cavitation is dependent upon the epithelial junctional complex and the cortical

cytoskeleton, perhaps due to their role in maintaining the basolateral distribution of the Na/K-ATPase and polarity of other transporters as well. ZO-1 polypeptides are not detectable by immunofluorescence in early cleavage stage bovine embryos until the morula stage in differentiating outer blastomeres (132). The ZO-1 distribution pattern consists of a thin band confined to the apical contact points between adjacent outer cells of morulae and blastocysts (132). These observations parallel findings in the early mouse embryo in which ZO-1 protein is first localized at contact sites between outer blastomeres following compaction (133). Recently it has been demonstrated that ZO-1 alpha+ isoform proteins first appear in compacting mouse morulae as perinuclear foci followed by membrane accumulation between the outer blastomeres (134). The establishment of zonular ZO-1 localization (59) and tight junction formation coincides with the onset of cavitation (133-135).

5 Na/K-ATPase AND BLASTOCYST FORMATION

Na/K-ATPase is a primary driver of Na^+ and water re-absorption in the kidney and is essential for the maintenance of body fluid and electrolyte homeostasis (136; see figure 3)). The enzyme establishes and maintains the high internal K^+ and low internal Na^+ concentrations found in eukaryotic cells (137-149). The enzyme actively transports 3 Na^+ out of the cell and 2 K^+ into the cell for each molecule of hydrolyzed ATP. The electrochemical gradient that the Na/K-ATPase generates is necessary for maintaining osmotic balance, the resting membrane potential, and the excitable properties of muscle and nerve cells (137-140). The Na^+ gradient provides the energy for Na^+-coupled transporters that mediate the translocation of ions (H^+, Ca^{2+}, Cl^-, PO_4^-), substrates (glucose and amino acids) and neurotransmitters (136). The catalytic alpha-subunit of M_r 100 kD is responsible for ion transport (139-145). The beta-subunit of M_r 40-60 kD facilitates the processing and insertion of the alpha-subunit into the plasma membrane (138, 145). In mammals these subunits are encoded by multi-gene families consisting of four alpha subunit genes (alpha1, alpha2, alpha3, alpha 4) and three beta subunit genes (beta 1, beta 2, beta 3), each displaying a
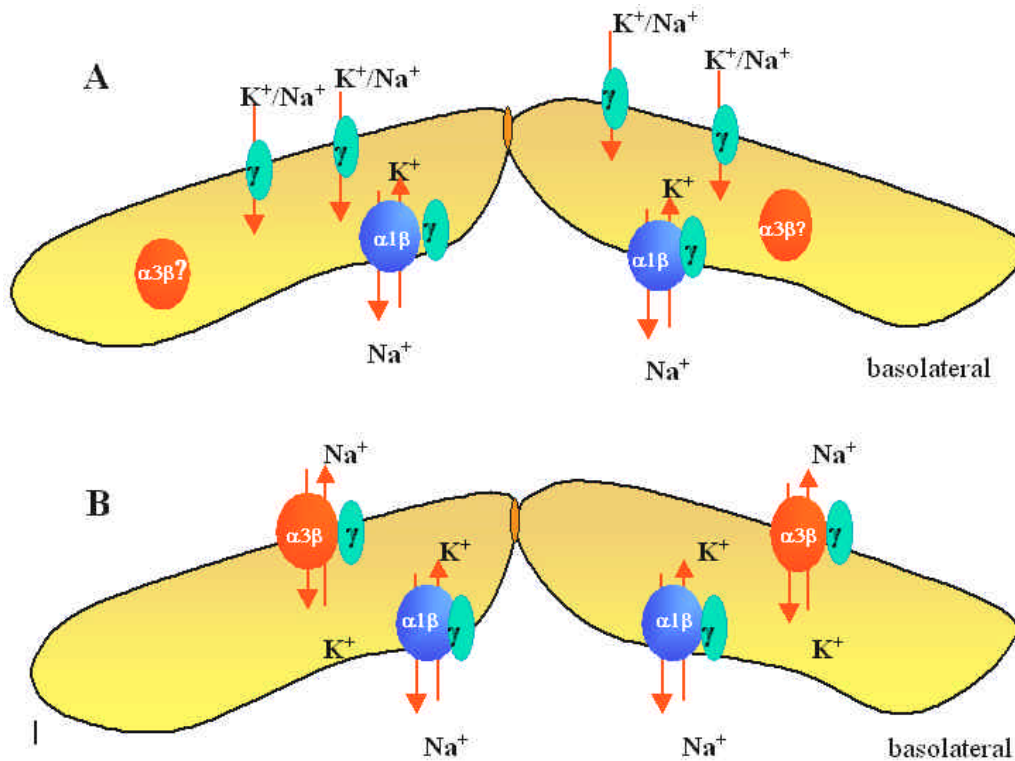
Figure 4. Differential expression of Na/K-ATPase subunit isoform protein in murine (A) and bovine (B) trophectoderm cells at the blastocyst stage.

distinct temporal and spatial expression pattern (139-146, 150-152). The gamma-subunit is a small (8-14 kD) hydrophobic peptide whose function is currently under investigation (146, 148, 150).

The alpha1/beta1-isozyme makes up the ubiquitous isozyme found in nearly every tissue and is the predominant kidney isozyme (137-149, 153). The alpha 2 and alpha 3 isoforms are present in low abundance in the kidney; the alpha 2 isoform predominates in adipocytes, muscle, heart and brain, while the alpha 3 isoform is most abundant in the brain (139, 153). The alpha 4 isoform is testes-specific (144). The beta 2-isoform is present in muscle, pineal gland and nervous tissue while the beta 3 isoform is present in testes, retina, liver and lung (154-156). **Why are so many Na/K-ATPase isoforms required?** The most logical view on their diversity is that differences in cation and ATP affinity of each isozyme are essential for adapting Na/K-ATPase activity to specific physiological conditions (157). Isozymes of Na/K-ATPase, composed of different combinations of alpha and beta subunit isoforms, have unique properties and are subject to developmental and hormonal regulation (158-175). For example, the choice of beta subunit does not greatly influence the kinetic properties of the enzyme but can have an effect on Na^+ affinity. Switching the alpha subunit significantly alters the affinity for Na^+, K^+ and ATP as well as the enzyme's sensitivity to ouabain (potent and specific inhibitor of Na/K-ATPase activity). In rodents, the alpha 1 isoform is ouabain resistant compared to other alpha isoforms (172-175).

5.1. Isoform expression in the mouse embryo

We initially detected Na/K-ATPase polypeptides by immunofluorescence at the late morula stage of mouse preimplantation development (62). Coincident with cavitation, the Na/K-ATPase immunofluorescence signal underwent a redistribution becoming restricted to the basolateral cell margins of the mural trophectoderm and its projections, overlying the inner cell mass (62, 83, 176). These early studies were applied to sectioned material, and no immunofluorescence was detected within the inner cell mass or polar trophectoderm. These findings were at variance with other studies where Na/K-ATPase activity was reported in mouse oocytes and early cleavage stages be measuring ion flux (49, 50) and also by measuring ouabain sensitive $^{86}Rb^+$ uptake in preimplantation stage embryos (53). We proposed that these methods were more sensitive than the immunofluorescence methods we initially employed and detected the " housekeeping" level of Na/K-ATPase activity present in all eukaryotic cells (177). We surmised that this low "housekeeping" level of the enzyme in the early cleavage stages increased in abundance to a level detectable by immunofluorescence in the late morula, just prior to the onset of cavitation (see figure 4). Additionally, Gardiner *et al.* (178) reported that both alpha and beta subunit polypeptides were detectable throughout preimplantation development by Western blot techniques.

The results from these early studies certainly suggested that isoforms of both alpha and beta subunits were present throughout preimplantation development (53, 178) and that by the blastocyst stage at least one isoform of the enzyme increased in abundance and became confined to the basolateral regions of the mural trophectoderm (62). These studies also raised the possibility that additional isoforms of the enzyme subunits may be expressed during early development.

Transcript levels for Na/K-ATPase subunit isoforms were first examined by probing three identical Northern blots with ^{32}P-labeled cDNAs encoding the three alpha-subunit isoforms of the rat Na/K-ATPase (66). The first reports characterizing the tissue distribution for the alpha 3-isoform within fetal brain and heart raised expectations that it may also be an "embryonic" isoform (139, 179-182). Northern hybridization experiments revealed the expression of alpha 1-isoform mRNAs throughout murine preimplantation development (66, 178). The hybridization signal displayed a pattern of mRNA expression that is typical for other mRNAs during mouse preimplantation development in which maternal mRNAs are subject to turnover and reduction in amount during the first cleavage division and later recover from the 2-cell stage, increasing in abundance through to the blastocyst (23-25, 28, 30, 35, 183). Watson et al. (66) reported a 43-fold decrease in Na/K-ATPase alpha 1-isoform mRNA between the zygote and 2-cell embryo stage. Gardiner et al. (178) reported a 45-fold increase in alpha-subunit mRNA from the 2-cell to the blastocyst stages. Following the 2-cell stage, the Na/K-ATPase alpha-1 subunit mRNA approximately doubles in abundance for every subsequent cleavage division, indicating that a relatively constant amount per cell is maintained (66).

Transcripts encoding the beta 1-subunit isoform accumulated in a very different manner throughout mouse preimplantation development (66). These transcripts were detected within zygotes but not in 2-, 4-, or 8-cell embryos by Northern blot analysis. Beta 1-subunit isoform mRNAs became detectable once again in morulae and underwent a further increase in abundance by the blastocyst stage (66). At first these results appeared to be at variance with the findings of Gardiner et al., (178) who reported the presence of the beta-subunit protein throughout preimplantation development by the technique of immunoblotting. Once again, the differences in sensitivity of methods used for these early studies proved to be the source of this apparent disparity. Both studies (66, 178) raised the possibility that the beta-subunit performs an essential role in coordinating the onset and progression of cavitation in the mouse embryo (see figure 4).

A more recent evaluation examining the presence of transcripts encoding the various subunit isoforms of the Na/K-ATPase employed the more sensitive reverse-transcription-polymerase chain reaction (RT-PCR) method and demonstrated that indeed additional isoforms of the Na/K-ATPase are expressed throughout murine preimplantation development (74). mRNAs encoding the alpha 2-isoform were detected in oocytes but not in any preimplantation embryo stage, while mRNAs encoding the alpha 3-isoform were detected in oocytes and in each stage of preimplantation development from the 2-cell to the blastocyst stage (74). mRNAs encoding the alpha 4-isoform were not detected in oocytes or during preimplantation development. In addition, this study reported that mRNAs encoding beta 2- and beta 3-isoforms were detectable throughout preimplantation development (74). These transcripts were not detected by Northern blot analysis (73), and thus do not likely accumulate to an appreciable level in the mouse. The presence of alpha 1- and beta 1-polypeptides were reported throughout murine preimplantation development by employing wholemount confocal microscopy methods. However, polypeptides encoding the alpha 3-, beta 2- and beta 3-subunits while expressed, were not localized to cell margins in any preimplantation stage. This study has clarified many of the earlier discrepancies by determining that multiple isoforms of the Na/K-ATPase subunits are expressed throughout murine preimplantation development. However, a number of questions remain as it is certainly not clear as to why multiple isoforms are expressed; how their membrane localization is regulated; and whether each expressed isoform contributes to the program of preimplantation development and cavitation in a unique manner or not.

Recently Jones et al., (148) reported that transcripts encoding the gamma-subunit accumulate from the 8-cell stage mouse embryo to the blastocyst stage. The gamma-subunit protein is present surrounding the surfaces of all blastomeres of the early mouse embryo including the trophectoderm and inner cell mass of the blastocyst. In contrast to alpha 1- and beta 1-subunit proteins, which become confined to the basolateral region of the trophectoderm, the gamma-subunit protein retains an apolar distribution in the trophectoderm (148). Antisense oligonucleotide inhibition of the gamma-subunit during early murine development resulted in an inhibition of ouabain-sensitive K^+ transport and a delay in cavitation independent of an effect on Na/K-ATPase expression (148). These experiments indicate that this subunit certainly contributes to the events of blastocyst formation but perhaps in a manner independent of simply modulating Na/K-ATPase activity (148). Further research is required to fully define the interactions of the gamma-subunit with the alpha and beta subunits during early development (see figure 4).

5.2. Isoform expression in the cow embryo

The presence of multiple isoforms of the Na/K-ATPase during preimplantation development was first reported by Betts et al (71) from studies applied to the bovine early embryo. By application of RT-PCR methods, transcripts encoding alpha 1-, alpha 2-, alpha 3-, and beta 2-isoforms of the Na/K-ATPase were detected throughout bovine pre-attachment development (71). Transcripts encoding the alpha 4-isoform were not detected in bovine embryos. As for the mouse embryo, this result was not unexpected as the expression of this Na^+-pump isoform is reported to be confined to the testes (144). mRNAs encoding the beta 1-isoform were first detected at the bovine morula stage and also in blastocysts (71). Detection
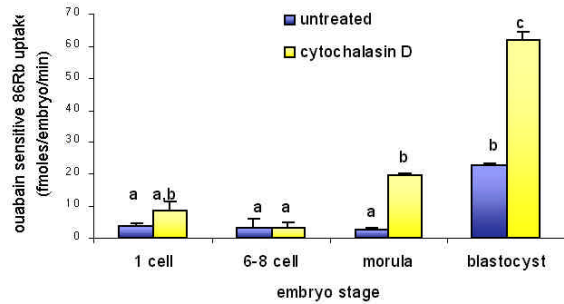
Figure 5. Pattern of detection of ouabain-sensitive ^{86}Rb$^+$ transport in *in vitro* produced bovine embryos treated either with or without 5 ug/ml cytochalasin D. At the morula and blastocyst stages, tight junction formation between embryonic blastomeres is demonstrated by increased ^{86}Rb$^+$ transport following disruption of cell-cell adhesion.

of beta 1-subunit mRNAs at the morula stage of bovine development agrees with the increase in the level of beta 1-subunit mRNA (66), beta 1 subunit protein (178), and enzyme activity (61) observed during murine preimplantation development. The absence of beta 1-subunit mRNAs in earlier bovine stages is distinct from that observed in the mouse and raises the possibility that other beta-subunit isoforms are responsible for the "house keeping" function of the enzyme in these stages. These results however, support the hypothesis that the timing of expression of the beta 1-subunit is closely coordinated with the onset of cavitation and may be important in regulating Na/K-ATPase activity and thus blastocyst formation. The beta 2-isoform is predominantly localized in the brain and nervous tissue. Its possible additional role in mediating cell adhesion (adhesion molecule of glia, AMOG) is of interest as it may provide a similar role during the first week of development (156, 184).

Na/K-ATPase isoform-specific antisera have been employed in whole mount indirect immunofluorescence assays to localize these polypeptides during bovine early development (55). Alpha 1-isoform polypeptides were localized to the basolateral cell margins of bovine trophectoderm and encircling each cell of the inner cell mass (ICM) (55). In contrast, alpha 3-isoform polypeptides were preferentially localized to apical cell surfaces of the trophectoderm and were not detected in the ICM (55). This is in contrast to studies applied to the mouse blastocyst, where alpha 3-isoform polypeptides were not localized to the trophectoderm cell margins in this species. Therefore, the localization of alpha 1-subunit polypeptides throughout bovine early development would agree with the findings for the murine early embryo. To date, alpha 3-subunit polypeptides have been primarily localized to neural tissue membranes (185). The localization of alpha 3-polypeptides to the apical trophectoderm surfaces in the bovine blastocyst was an unexpected finding. Therefore the possibility of both apical and basolateral Na/K-ATPase Na$^+$-pumps exists for bovine trophectoderm. The results in total indicate that the role of the Na/K-ATPase in blastocyst formation is more complex than first thought. They have revealed species-specific

differences including the presence of alpha 2-isoform transcripts throughout bovine early development and localization of alpha 3-subunit polypeptides to the apical domain of bovine trophectoderm cells (see figure 4). The specific contributions of each Na/K-ATPase subunit isoform to this blastocyst formation are not known but are the subject of present studies**.**

5.3. Ouabain sensitivity and Enzyme activity

Direct evidence supporting a role for Na/K-ATPase in blastocyst formation comes from ouabain studies (ouabain is a specific inhibitor of the enzyme) (63, 149, 157). Ouabain-sensitive primate cells, such as CV-1 African green monkey cells, are sensitive to 5 x 10^{-8} M ouabain, while ouabain-insensitive murine cells can survive treatment with ouabain at concentrations of 5 x 10^{-4} M (186, 187). Blastocyst expansion is restricted in the presence of ouabain and blastocyst re-expansion following cytochalasin-induced collapse is completely blocked in the presence of ouabain (9, 63, 69, 70). Dizio and Tasca (63) treated cytochalasin B-collapsed blastocysts with 10^{-3} M ouabain, while Wiley (9) demonstrated an effect on cavitation when concentrations of at least 10^{-4} were used. Van Winkle and Campione (53) were unable to detect more than a two-fold increase in ouabain sensitive ^{86}Rb$^+$ uptake throughout murine preimplantation development when the increase in cell surface area between oocytes and blastocysts was considered. These results suggest that, although ouabain sensitive enzyme activity may not increase dramatically during murine preimplantation development, the presence of immunofluorescently detectable protein certainly does. Ouabain inhibition of Na/K-ATPase also promotes the exchange of intracellular K$^+$ for extracellular Na$^+$ in murine embryos demonstrating that Na/K-ATPase activity maintains steep Na$^+$ and K$^+$ gradients in embryonic cells just as it does for other cells (47). We have demonstrated that bovine blastocysts are more sensitive to ouabain than murine blastocysts (71). We measured ouabain-sensitive ^{86}Rb$^+$ transport and therefore Na$^+$-pump activity throughout bovine pre-attachment development in cytochalasin D treated embryos (55; see figure 5). Cytochalasin treatment is employed to disrupt trophectoderm tight junctions to allow ouabain access to Na$^+$-pumps localized to the basolateral membranes of the trophectoderm (71). ^{86}Rb$^+$ uptake was largely maintained at a constant level from the 1-cell zygote to the 6-8 cell stage, but then increased dramatically to the bovine blastocyst stage. Increased ouabain-sensitive ^{86}Rb$^+$ uptake parallels the increased abundance of Na/K-ATPase subunit mRNAs, subunit proteins, and enzyme activity observed during murine and bovine blastocyst formation. In contrast to the two-fold increase in ouabain-sensitive ^{86}Rb$^+$ uptake reported for murine embryos throughout the first week of development (53), we observed over a 18-fold increase in uptake from the 6-8 cell to bovine blastocyst stages (cytochalasin treated groups) (55). In addition, in contrast to findings in the murine blastocyst considerable ouabain-sensitive ^{86}Rb$^+$ uptake was measured in bovine blastocysts not subjected to cytochalasin treatment (55). Van Winkle and Campione (53) also reported ^{86}Rb$^+$ transport within intact murine blastocysts raising the possibility that a component of this Na/K-ATPase transport activity may be

directed from the apical trophectoderm membrane of mammalian blastocysts. Alternatively, the junctions between trophectoderm cells might be leaky, allowing the paracellular movement of $^{86}Rb^+$ and ouabain into the blastocyst cavity.

5.4. Role of Na/K-ATPase in Cavitation

Incubation of mouse embryos with antiserum raised against E-cadherin prevents normal cavitation (67). Treated embryos contained a variable number of intra-blastomeric fluid-filled cavities, which were encircled with Na/K-ATPase immunofluorescence (67). Under these treatment conditions the trophectoderm apical junctional complex was disrupted and embryos were observed in which (those without extensive fluid-filled cavities) the cytoplasm of some blastomeres became densely packed with smaller vesicles, which were possibly the origin of the larger fluid-filled cavities (67). These results suggest that the targeting of the nascent Na-pumps to the appropriate trophectoderm cell membranes was impaired thus confining the Na/K-ATPase within cytoplasmic secretory vesicles. This, combined with activity of the enzyme, may result in the osmotic accumulation of water in the vesicles to form intracellular fluid-filled cavities (52, 67). A similar effect occurs when embryos are cultured in medium with minimal calcium (78). Geering *et al.* (188) suggested that the nascent catalytic subunit of Na/K-ATPase aligns with the beta-subunit within the membranes of the rough endoplasmic reticulum (RER) and golgi en route to the plasma membrane. The nascent enzyme could undergo cation-dependent conformational changes under these conditions (188, 189). Normally, (as observed by the immunoreactivity in untreated morulae), the nascent enzyme would not begin to transport Na$^+$ and K$^+$ prior to its insertion into the plasma membrane. These findings certainly linked the normal targeting and insertion of the Na/K-ATPase to the trophectoderm basolateral cell margins with normal blastocyst formation.

The relative insensitivity of mouse cavitation to ouabain (sensitive to 10 $^{-3}$M ouabain) implies that the more ouabain sensitive alpha 2- and alpha 3-isoforms are not likely the predominant isoforms. In contrast to mouse embryos, bovine embryos are extremely sensitive to ouabain (10 $^{-7}$-10 $^{-9}$M) raising the possibility that the alpha 1-isoform is not the predominant alpha-subunit in early embryos of this species. The immunofluorescence localization of the alpha 3-subunit to the apical trophectoderm membrane domains in bovine blastocysts may in part, explain their greater ouabain sensitivity when compared to their murine counterparts. In addition, bovine blastocysts can be collapsed simply by treatment with high (10^{-6}M to 10^{-3}M) ouabain, without the initial treatment with cytochalasin D (55). These data support the possibility that the apical alpha 3-subunit plays an active role in blastocyst formation in the bovine embryo.

The detection of multiple subunit isoforms, and varying species sensitivities to ouabain, raise questions such as: What roles do various isozymes of the sodium pump have during early development? Do the varying ouabain sensitivities displayed by these species reflect comparative variations in the abundance of alpha-isoforms? Different isoforms display distinct ouabain sensitivities and ion affinities, and Na$^+$ and K$^+$ concentrations can influence isoform expression. Jewell and Lingrel (169) found that rat alpha 1- and alpha 2-isoforms in HeLa cells have similar apparent affinities for Na$^+$, but that the alpha 3-isoform has a 4-fold lower affinity for this ion. Low K$^+$ concentrations decrease alpha 2-subunit mRNA levels and protein levels by 38% and 82%, respectively, in hypokalemic skeletal muscle (190). Although vectorial transport of Na/K-ATPase to the basolateral surface of membranes is conventional (191) apical localization of the sodium pump is reported for several tissues (192, 193). Immunofluorescence studies do not shed any light on the functional significance of alpha 1- and alpha 3-subunit polypeptides to the overall mechanism of cavitation, although treatment of collapsed bovine blastocysts with 10^{-9} M ouabain causing reduced blastocyst re-expansion is highly suggestive of the alpha 3-isoform contributing to blastocyst formation (71). We hypothesize that an apical trophectoderm alpha 3-isoform could participate in regulating the steepness of the trans-trophectoderm Na$^+$ gradient required to drive the osmotic accumulation of water across this epithelium to form the blastocyst cavity. Support for this role comes from studies that report the alpha 3-isoform may regulate high intracellular Na$^+$ loads in order to restore physiological Na$^+$ levels (194). We are uncertain as to how high trophectoderm cell intracellular Na$^+$ levels rise during blastocyst formation. Baltz *et al.*, (47) measured intracellular Na$^+$ and K$^+$ levels up to the murine morulae stage. We propose that the hypothesized increase in intracellular Na$^+$, required to drive blastocyst formation, must be regulated. An apical alpha 3-isozyme of the Na/K-ATPase could perform this role and maintain the trophectoderm cell Na$^+$ gradient within physiological limits. Since the alpha 3-subunit displays a much lower affinity for cytoplasmic Na$^+$ than the alpha 1-subunit (175), the presence of an apical alpha 3-subunit would not necessarily disrupt a trans-trophectoderm Na$^+$ gradient (driven by the basolateral Na/K-ATPase), but instead could serve to modulate the gradient by moving Na$^+$ from inside the cell to the extra-embryonic environment. Functional studies applied to isoform specific null mutants are required to determine the precise ion transport properties of each Na/K-ATPase isoform during blastocyst formation.

We have initiated studies employing antisense oligonucleotide inhibition of Na/K-ATPase isoform expression during bovine early development. Bovine zygotes have been cultured with alpha 1-isoform specific antisense phosphorothioate oligodeoxynucleotides (ODNs) to disrupt translation of this protein. Preliminary results have demonstrated that treatment of bovine 6-8 cell embryos with alpha 1-antisense ODNs significantly impairs blastocyst formation (see figure 6). It has however been very difficult to standardize this effect of alpha 1-isoform ODNs on blastocyst formation. Therefore examining the consequences to early development following the targeted deletion of Na/K-ATPase subunit isoforms represents a more viable route for discerning the specific contributions of each isoform to blastocyst formation.
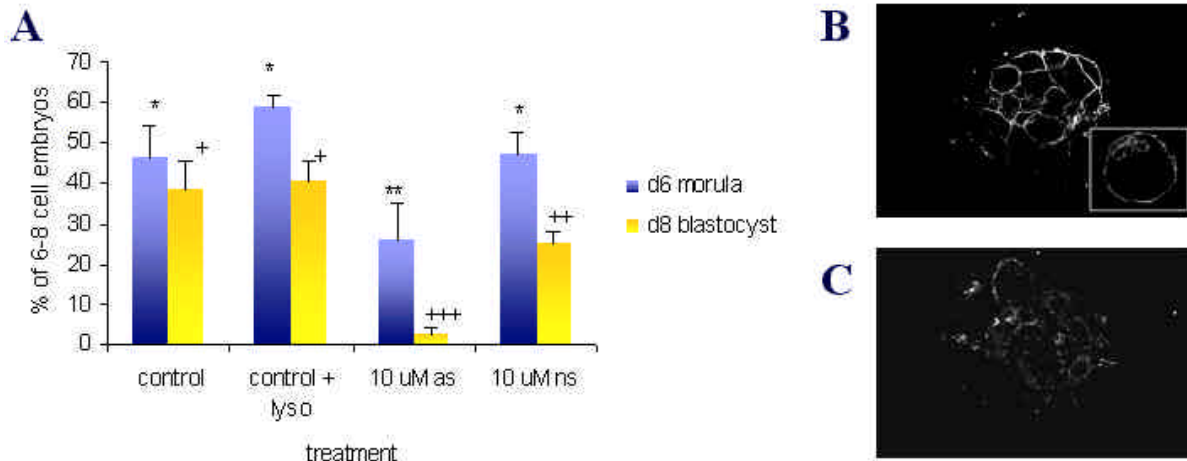
Figure 6. Development of bovine embryos treated with 10 u M alpha 1-subunit antisense oligonucleotides *in vitro* (A). Antisense treatment significantly reduced development to the blastocyst stage (n=5 replicates of 20 embryos; p<0.05). When alpha 1-subunit protein expression was compared to control embryos (B), alpha 1 antisense treated embryos demonstrated a marked reduction in immunofluorescence signal (C).

5.5. Murine Knock-outs

Several studies have reported outcomes from gene targeting experiments that have "knocked-out" the expression of specific Na/K-ATPase subunit isoforms. Studies from Dr. Jerry Lingrel's laboratory (University of Cincinnati) have demonstrated that alpha 1 subunit null mutants are never born and must suffer a developmental lethality (the precise time of their demise is unknown), while alpha 2-subunit null mutants are born but do not survive the first day (195). Alpha 2-isoform null mutants have also been generated by homologous recombination and these mice suffer no ill effects until after birth (154). Other isoforms have not been targeted as yet but the findings would suggest that the alpha1-subunit is the predominant isoform required for development while the beta 1-isoform is the most reasonable candidate for regulating the expression of functional enzyme units in the trophectoderm. Experiments examining the timing of lethality for the alpha 1-subunit null mutants are currently underway and it is expected that this murine line will prove useful for defining the role of the alpha 1-isoform during blastocyst formation.

6. WATER TRANSPORT AND BLASTOCYST FORMATION

Transporting epithelia are characterized by the rapid passage of water across the cell layer (196-199). However, the mechanisms that facilitate the movement of water across the trophectoderm are largely unexplored. The processes that regulate the uptake and release of water by living cells are fundamental to cell viability. Water can diffuse through lipid bilayers, and all cell membranes display some level of permeability to water (for review see (196, 197, 200, 201)). The diffusional permeability (P_d) of cell membranes varies but is usually small ie. < 1 m min $^{-1}$ atm $^{-1}$ (196, 198, 202). Although this value is low, it is sufficient for most cell types to regulate their volumes. Thus, it was widely believed that simple diffusion was the primary if not exclusive mechanism for mediating water

movement through biological membranes and that water channels were not necessary (201). Water flow in some cell types such as red blood cells is, however, much greater than that predicted by simple diffusion alone (203). This has resulted in the hypothesis that cell membranes possess channels that facilitate water flow (196-199). Evidence supporting the presence of "water channels" has come from biophysical studies of red blood cells, kidney collecting ducts and toad bladder epithelial cells (200. 204-207; see figure 7). Water permeabilities across these membranes are 10- to 100-fold greater than that observed in phospholipid bilayers. The ratio of osmotic permeabilities (P_f, determined by the measurement of bulk water flow in response to an osmotic gradient) to diffusive permeabilities (P_d, measurement of the flux of isotopic water under conditions of equal osmolality) are far greater than 1 (ratio observed in lipid bilayers) (196-213). This increased ratio is characteristic of water channel-mediated fluid transport. Extensive research conducted largely over the past ten years has indicated that plasma membranes display a varied permeability to water movement and that in cases of rapid and large volume water movement specific water transport molecules must exist (for review see (196, 197, 200, 201). These types of studies have resulted in the characterization of a family of 10 molecular water channels or Aquaporins. We hypothesize that AQP water channels are present in the trophectoderm cells of the preimplantation embryo and that they function as conduits for the trans-trophectodermal fluid movements associated with blastocyst formation.

6.1. Aquaporins

Aquaporins (AQPs) belong to a family of major intrinsic membrane (MIP) proteins, and function as molecular water channels that allow water to flow rapidly across plasma membranes in the direction of osmotic gradients (see figure 8). Ten different AQPs have been identified in mammals (197, 205-207, 209, 214).

AQP 0/MIP28 was isolated from lens epithelium (215). Absence of normal AQP 0 protein is linked to
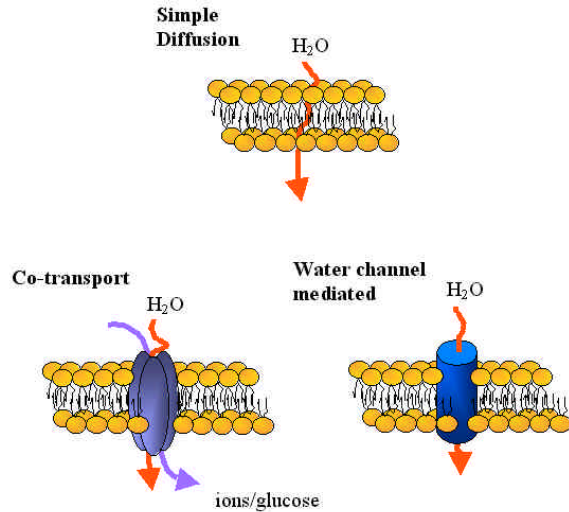
Figure 7. Mechanisms of water movement across biological membranes. Water generally crosses the lipid bilayers by i) simple diffusion, ii) co-transport during the transport cycle of enzymes and other transporters or iii) through pores created by the expression of aquaporins.
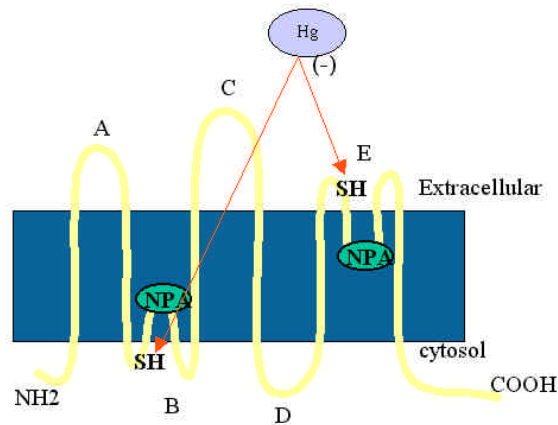

Figure 8. Theoretical membrane structure aquaporin proteins. AQP proteins consist of 6 membrane spanning domains and 5 loop domains, of which infoldings of loops B and E are believed to form the water pore and possess SH-groups which when bound by mercury result in inhibition of aquaporin function.

cataract formation in mutant mice (215). AQP 1/channel forming integral membrane protein (CHIP)-28 is the most ubiquitous of the AQP family and is expressed in a number of organs and tissues (216-219). AQP 1 is the major water channel in red blood cells and in the brain it is only found in the choroid plexus (216-219). AQP 2 is the vasopressin responsive water channel found in kidney collecting ducts (220-222). This water channel is stored in cytoplasmic vesicles that fuse with the apical membrane following exposure to vasopressin (221). Loss-of-function mutations in this gene result in nephrogenic diabetes insipidus (223). AQP 3 permits the passage of water, glycerol and urea and is generally located to the basolateral membrane of epithelial cells (222-225). AQP 4 is a mercury insensitive

water channel also generally localized to the basolateral membranes of a variety of epithelial cells (226-231). AQP 5 is localized to apical membranes, and is involved in the secretion of saliva, tears and pulmonary fluid (232, 233). Murine null mutants for AQP 5 display a marked reduction in saliva production (233). AQP 6 has a low water conducting ability and its expression may be confined to the kidney (234). AQP 7 permits the passage of both water and glycerol and is expressed in a transient fashion in late spermatids and maturing sperm and weakly in kidney and heart (235). AQP 8 is also localized in the testes as well as placenta, colon, liver, and heart (236). AQP 9 is the most recently cloned water channel and is most similar to AQP 3 and 7 in that it also permits glycerol transport. It is expressed in leukocytes and liver and is weakly expressed in the heart, kidney and small intestine (224,237, 238). The blastocyst provides a comparatively simple system from which fundamental information on AQP gene expression and function can be obtained.

6.2. Aquaporin Expression and Function During Blastocyst Formation

Two studies have examined the presence of aquaporin (AQP) family transcripts during murine preimplantation development. The first by Edashige *et al*., (239) reported the detection of mRNAs encoding AQPs 3, 7, 8 and 9 in preimplantation stages. mRNAs encoding AQPs 1, 2, 4, 5, and 6 were not detected in any stage. Interestingly mRNAs encoding AQPs 8 and 9 were only detected in blastocysts. Offenberg *et al*., (240) conducted a similar study but reported that mRNAs encoding AQPs 1, 3, 5, 6, 7, and 9 were detectable in all stages from 1-cell zygotes to the blastocyst stage. In addition, AQP 8 mRNAs were detected only in morula and blastocyst stages. Although Offenberg *et al*., (240) did not employ a semi-quantitative analysis it was noted that for two additional AQPs (AQP 3 and 7) there was a consistent increase in signal in morula and blastocyst stage embryos suggesting that mRNAs encoding AQPs 3, 7, and 8 may be upregulated in conjunction with blastocyst formation. Confirmation of this possibility however must be made by applying semi-quantitative methods of analysis. The findings from these two studies vary somewhat. The differences observed between these two studies may be related to primer design or amplification efficiency differences or perhaps to differences in starting total mRNA levels or size of embryo batches employed. Never the less these studies do agree that the mRNAs encoding several AQPs are expressed during preimplantation development and that for at least one AQP (AQP8) expression is coincident with blastocyst formation.

We have also examined the expression of mRNAs encoding AQPs during bovine early development and found that mRNAs encoding AQP 3, 5, 8 and 9 are detectable in bovine blastocysts and control tissues. The absence of bovine AQP sequence data in the GenBank has hampered the design of effective primer sets for this species and may account for the difference in AQP family member expression between mouse and bovine embryos. These results indicate that several AQP gene products are expressed during murine and bovine blastocyst formation.
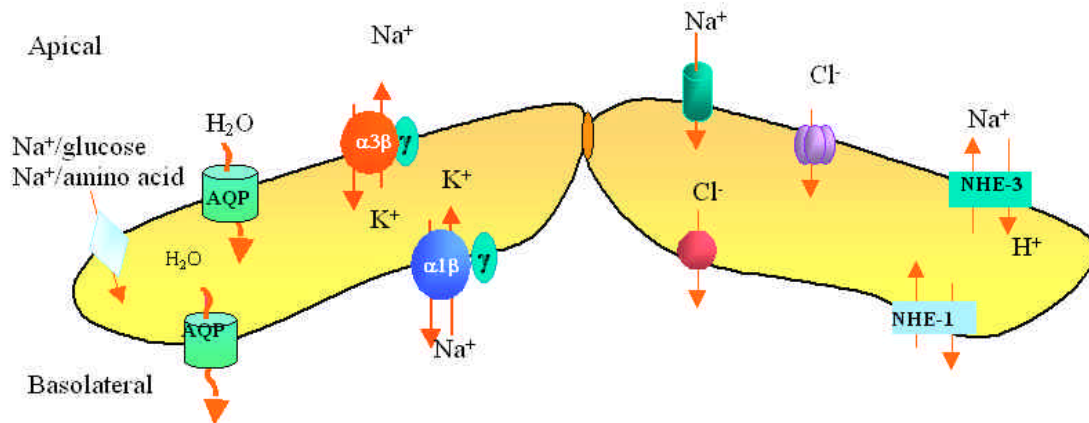
Figure 9. Model of ion and fluid transport across the trophectoderm of the mammalian blastocyst.

We have also initiated experiments to contrast the distribution of AQP polypeptides throughout murine early development by confocal immunofluorescence microscopy. Preliminary results indicate that AQP 3, 6, 8, and 9 polypeptides are present in murine blastocysts with AQP 3 polypeptides localized to trophectoderm basolateral domains, AQP 8 and 9 polypeptides display an apical and basolateral localization pattern and AQP 6 polypeptides display a cytoplasmic localization. Preliminary results assessing sensitivity of blastocyst expansion to treatment with inhibitors following exposure to hypo-osmotic medium also indicate a role for AQP water channels in blastocyst formation.

Future experiments will investigate the functional roles of these AQPs to blastocyst formation and will localize each expressed AQP during early development as effective antisera for each AQP become available. The pursuit of functional data is hampered by the absence of specific AQP inhibitors. The existence of AQP null mutant murine lines and the application of antisense oligodeoxynucleotide approaches should enable the pursuit of the individual and collective roles that AQPs play during early development. We expect that much more information regarding the role of AQPs in regulating blastocyst formation will emerge over the next few years.

7 . SUMMARY AND FUTURE PROSPECTS

There is a great need to understand the mechanisms controlling mammalian preimplantation development in order to develop rational interventions to either promote or inhibit fertility (3,5, 241, 242). Current research is addressing this need and is aimed at providing an eventual understanding of the causative events underlying early embryo loss and in ensuring that assisted reproductive technologies are applied in a safe and efficient way. The results from these studies impact on our understanding of basic transport biology and physiology, epithelial (trophectoderm) cell differentiation and blastocyst formation and thus improve our ability to understand human infertility, early embryo loss and epithelial cell disease states.

Blastocyst formation is dependent upon the expression of several gene products including Na/K-ATPase subunits, E-cadherin, and tight junction components (see figure 9). This morphogenetic event is initiated during compaction as stable E-cadherin/catenin cell-to-cell adhesion junctions form as a prelude to focal tight junction formation. Trophectoderm differentiation proceeds as tight junctions mature and assist in the establishment of distinct apical and basolateral cell membrane domains. By the late morula stage, the beta 1 subunit of the Na/K-ATPase is upregulated and this occurs in conjunction with the polarized localization of alpha 1 isoforms to the basolateral domains of the outer trophectoderm, at the onset of fluid accumulation. In the bovine embryo, a second alpha 3-isoform is expressed and assumes a predominantly apical localization pattern. It is hypothesized that the blastocyst cavity formation is facilitated by a trans-trophectoderm ion gradient established, in part, by a polarized trophectoderm Na/K-ATPase, which facilitates the osmotic movement of water into the extracellular space to form the fluid-filled cavity of the blastocyst. The apical alpha 3 isoform in the bovine trophectoderm may assist in regulating the steepness of the Na^+ gradient across the epithelium. Blastocyst expansion only occurs when the tight junction permeability seal fully forms to restrict the leakage of fluid via paracellular routes, ensuring the expansion of the cavity as fluid accumulates. The mammalian blastocyst also expresses mRNAs encoding several AQPs. These water channels may direct the rapid movement of water across the epithelium to form the blastocyst fluid. The precise roles of Na/K-ATPase isoforms and AQP water channels during blastocyst formation have not been defined. Future research will be directed at defining their individual and collective roles in supporting blastocyst formation.

ACKNOWLEDGMENTS

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