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Maternal embryokines that regulate development of the bovine preimplantation embryo

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Abstract: Proper embryonic development depends upon regulatory signals from the reproductive tract called embryokines. This review uses the cow as a model to understand the developmental processes controlled by embryokines. The focus is on 3 embryokines that have been shown to increase competence of embryos to survive after transfer to recipients: CSF2, IGF1, and DKK1. Together, these molecules regulate key events required for development of the embryo to the blastocyst stage including proliferation (enhanced by IGF1), development of the inner cell mass (regulated by CSF2), control of differentiation (modified by DKK1), and inhibition of apoptosis and stress-mediated developmental arrest (IGF1 and CSF2).

Key words: Embryo, embryokine, colony stimulating factor 2, insulin-like growth factor-1, dickkopf 1

1. Importance of maternal signals for development of the bovine blastocyst

The zygote contains within its genome and epigenome the program to direct development through the embryonic, fetal, and postnatal periods. The execution of that program is dependent on the embryo's nongenetic inheritance from the oocyte and sperm (1,2) as well as on the environment in which development proceeds. Variation in the maternal environment can affect the ability of the preimplantation embryo to establish pregnancy. In cattle, the focus of this review, competence of the preimplantation embryo for growth and survival can be affected by maternal parity (3), lactational status (4), and circulating concentrations of progesterone (5). Moreover, patterns of gene expression in the endometrium are associated with survival of an embryo transferred to the uterus in the next ovulatory cycle (6,7).

Development of the bovine embryo to the blastocyst in the absence of maternal signals (i.e. through in vitro production procedures) results in embryos with aberrant gene expression (8,9), lipid content (10,11), DNA methylation (12), and, as shown in the Table, reduced competence to establish pregnancy after transfer into recipients (13–17). Additionally, an increased proportion of embryos produced in vitro have developmental abnormalities that lead to increased neonatal death losses (18–20). Some of the problems with the in vitro-produced embryo could result from selection of incompetent oocytes or inadequate oocyte maturation. However, the importance of the maternal environment during development is indicated by observations that transfer of in vitro-produced embryos to the oviduct after fertilization limits some of the abnormalities associated with in vitro production (9,21).

One function of the reproductive tract is to secrete bioactive molecules that regulate the embryo, oviduct, or endometrium. Genes for 115 ligands expressed in the endometrium had the corresponding receptor gene expressed by the embryo (22). Regulatory molecules produced by the oviduct and endometrium, which include hormones, growth factors, and cytokines, are referred to as embryokines when they function to regulate embryonic growth and development (23).

It is likely that some of the variation in the ability of the reproductive tract to support embryonic development represents variation in secretion of embryokines. Indeed, several genes that were overexpressed in the endometrium of cows that subsequently became pregnant after embryo transfer as compared to cows that did not establish pregnancy are potential embryokines. These include *NGF*,

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Table. Differences in pregnancy rate after transfer of fresh embryos produced in vivo or in vitro.

Recipient type	Pregnancy rate			Reference
	Day of pregnancy diagnosis	Embryos produced in vivo	Embryos produced in vitro	
Heifers	53	79% (n = 19)	37% (n = 19)**	13
Beef and dairy heifers	50	55% (n = 199)	37% (n = 90)**	14
Bos taurus × *B. indicus* heifers	60	42% (n = 289)	34% (n = 910)*	15
Bos taurus × *B. indicus* nonlactating cows and heifers	30	59% (n = 90)	31% (n = 87)***	16
Holstein cows, nonlactating	98	50% (n = 115)	43% (n = 145)	17

*: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$.

VEGFB, and *WNT11* (6) and *CSF3*, *CXCL2*, *IL18*, *IL1B*, and *TNFSF13* (7). Among the genes upregulated in heifers of high fertility versus those of lower fertility were *CXCL2*, *DKK1*, and *SFRP4* (24).

Despite the importance of secretory molecules from the reproductive tract for control of early embryonic development, little is known about how specific embryokines regulate development of the embryo. The purpose of this review is to use the bovine as a model to understand the developmental processes controlled by embryokines that increase competence of the embryo to develop successfully to term. Focus will be on those embryokines in which effects on competence of embryos to survive after transfer to recipients have been examined: CSF2, IGF1, and DKK1. These molecules are not the only maternally derived regulatory molecules that can affect preimplantation development in the bovine. Other examples include activin (25,26), ILB1 (27), LIF (28), TGFB (28), FGF2 (28,29), and EGF (30). However, there is sufficient information about the role of CSF2, IGF1, and DKK1 on the bovine embryo to allow identification of functions regulated by these molecules that are likely important for developmental competence of the embryo. Among these functions is stimulation of proliferation, development of the inner cell mass (ICM), control of differentiation, and inhibition of apoptosis and stress-mediated developmental arrest.

2. Colony stimulating factor 2

Also called granulocyte-macrophage colony stimulating factor, CSF2 was discovered as a hematopoietic growth factor that controls development and function of granulocytes and macrophages (31). The cytokine is produced by several cell types, usually in response to an inflammatory signal, including fibroblasts, smooth muscle cells, endothelial cells, and monocytes (32). Moreover, CSF2 acts on a variety of cells to stimulate growth, block apoptosis, regulate chemotaxis, and induce release of bioactive molecules (33).

One of the sites of synthesis of CSF2 is the oviduct and endometrium, with greatest localization in the luminal epithelium (34–36). There is little cyclic variation in labeling intensity of CSF2 in the endometrium (34,35). Pregnancy may modify secretion of CSF2 because intensity of immunolabeling in the endometrial stroma was increased at day 7 in pregnant cows as compared to cyclic cows and labeling in the luminal epithelium of the endometrium was increased by intrauterine treatment with the embryonic secretory product IFNT (35). Immunoreactive protein and mRNA for *CSF2* in the oviduct was reduced in obese cows (36), so environmental factors may also modify secretion of the cytokine.

In the cow (37,38), as well as in the mouse (39) and human (40), treatment of embryos in vitro with CSF2 increases the proportion of embryos that survive after transfer to recipient females. Results of experiments with cattle are summarized in Figure 1. CSF2 increased the percent of recipients diagnosed as pregnant at 30–35 days of gestation when the cytokine was administered from day 5 to 7 of development (i.e. when the embryo was a morula or blastocyst), but not when treatment was from day 1 to 7 after development (i.e. from the zygote to blastocyst stage). It is not really known whether the duration of CSF2 treatment is crucial for the change in embryonic function leading to increased pregnancy rate. That it may not be is indicated by the observation that CSF2 reduced fetal loss after initial pregnancy diagnosis when embryos were exposed to CSF2 from either day 5 to 7 (in 1 of 2 experiments examined) or day 1 to 7. What is more likely is that the small sample size for each experiment created some variability in response.

There are several changes in the embryo caused by CSF2 that could potentially be responsible for the increased competence to establish pregnancy. Blastocysts produced

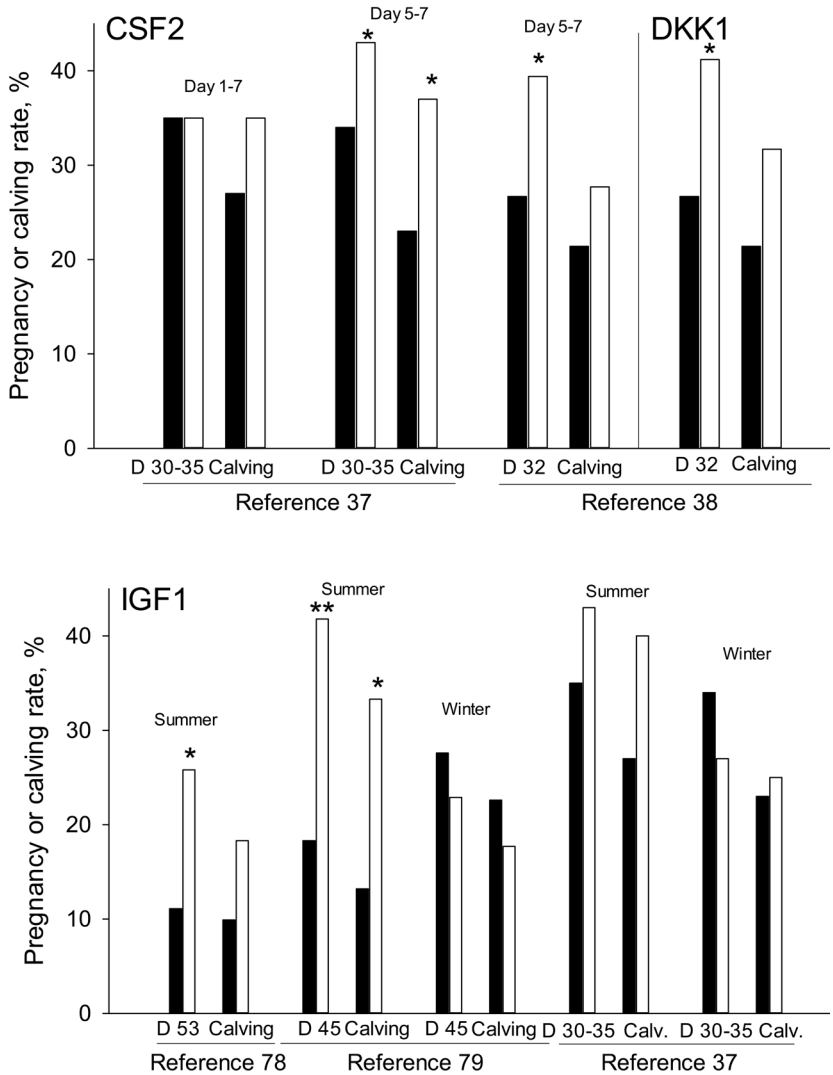

Figure 1. Culture with CSF2, IGF1, and DKK1 improves survival of in vitro-produced embryos after transfer into recipients. Data represent pregnancy and calving rates. Black bars represent cows receiving control embryos while open bars represent cows receiving embryos treated with CSF2 from day 1 to 7 or 5 to 7 of development, DKK1 from day 5 to 7 of development, or IGF1 for the first 7 or 8 days of development. Significant differences are indicated by asterisks (*: $P < 0.05$; **: $P < 0.01$).

in the presence of CSF2 have increased numbers of cells in the ICM (37). Moreover, CSF2 improves survival of isolated ICM maintained in culture (41). These actions of CSF2 on the ICM could be important for embryonic survival since 25% or more of in vitro-produced embryos at days 14–17 of gestation are reported to be without the embryonic disk derived from the ICM (42–44). CSF2 also has actions on development of the trophoblast because embryos collected at day 15 have more extensive development of extraembryonic membranes when exposed to CSF2 from day 5 to 7 of development than when not treated with CSF2 (44). This action of CSF2 depends on sex, with CSF2 increasing the length of male embryos and decreasing the length of female embryos (45).

Some of the actions of CSF2 on the embryo could be mediated by inhibition of apoptosis. Culture with CSF2 altered expression of several genes involved in apoptosis and reduced the increase in apoptosis caused by exposure of embryos to heat shock (46). Interestingly, CSF2 increases the percent of embryos becoming blastocysts only when development in control embryos is low (41). This result could indicate that CSF2 is reversing some aspects of cellular stress that lower development.

CSF2 also altered expression of 42 genes involved in the developmental process ontology of DAVID, including several genes whose change in expression was interpreted to indicate promotion of mesoderm formation and regulation of WNT signaling (46).

The prototypical signal transduction system for CSF2 involves a low-affinity α subunit (CSF2RA) and a high-affinity β subunit (CSF2RB) (47). In the cow (41), as in other species (48–50), *CSF2RA* is expressed in the preimplantation embryo but expression of *CSF2RB* is undetectable or nearly so. It is likely, therefore, that CSF2 signaling in the embryo involves an unknown pathway that is independent of CSF2RB. In *Xenopus* oocytes, signaling through CSFRA is possible through a mechanism involving H_2O_2 generation and phosphatidylinositol 3-kinase (51).

3. Insulin-like growth factor 1

Several features of its biology make it difficult to understand the actions of IGF1 in the reproductive tract. The gene itself is subject to differential splicing and the protein can be modified after translation by proteolysis and glycosylation (52). IGF1 is part of a larger family of proteins including INS and IGF2, and each can interact with each other's receptors (INSR, IGF1R, and IGF2R) (53). The effective concentration of IGF1 and IGF2 for receptor interactions depends upon concentrations of 6 separate IGF-binding proteins, which prevent receptor binding while also increasing the half-life of IGF1(52).

IGF1 is both a hormone, being released by the liver to mediate actions of somatotropin, and a locally produced growth factor. Accordingly, IGF1 concentrations in the lumen of the reproductive tract depend upon local synthesis and transudate from the blood. The extent to which changes in circulating concentrations of IGF1 modify local amounts in oviductal or uterine fluids is not well established. There were nonsignificant increases in amounts of IGF1 in uterine lumen of cows in response to administration of bovine somatotropin (54,55).

In the oviduct, local expression of *IGF1* occurs in the endosalpinx of the ampulla and isthmus (56,57), particularly in the stroma underneath the luminal epithelium (56). Protein localization, in contrast, is greatest in the luminal epithelium (57,58). Expression peaks around estrus and declines thereafter (56,57). *IGF1* is also expressed in the uterine endometrium (55,59,60). There is little variation in expression between days 5 and 16 after estrus in cyclic or pregnant cows (61). Endometrial expression of *IGF1* was not regulated by somatotropin in 2 experiments (59,60). In a third study, expression of *IGF1* as well as *IGFBP2* and *IGFBP3* was increased in endometrium of cyclic but not pregnant animals at day 17 after estrus (55).

The bovine embryo expresses *IGF1R* during early development, with steady-state amounts of *IGF1R* mRNA decreasing from the zygote stage to day 3 after fertilization and then increasing steadily to the blastocyst stage (62). Two major signal transduction systems activated by binding of IGF1 to the IGF1R are the MAPK pathway, which leads to increases in proliferation, growth, and differentiation, and the PI3K/AKT pathway, which inhibits apoptosis (63). Actions of IGF1 on the preimplantation embryo indicate that both MAPK and PI3K/AKT pathways are activated (Figure 2). Culture with IGF1 increases the percent of embryos capable of developing to the blastocyst stage (28,30,64–69). This action of IGF1 probably involves increased proliferation because the cell number of day 6 morulae was increased by IGF1 (70). Effects on development could be blocked with antibody to IGF1R (64) and an inhibitor of MAPK activation (69,70). IGF1 also blocks activation of apoptosis caused by exposure to heat shock (70,71) or the prooxidant menadione (72). Antiapoptotic actions of IGF1 in heat-shocked embryos

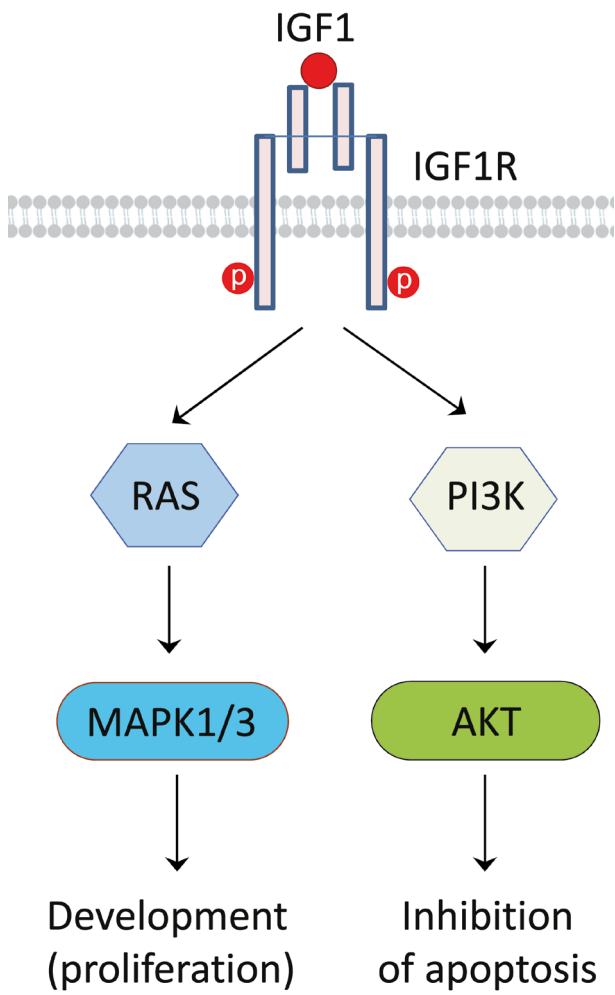

Figure 2. Signal transduction pathways for actions of IGF1 on the preimplantation bovine embryo. Studies with chemical inhibitors indicate actions of IGF1 on cell number and competence to develop to the blastocyst stage are mediated by the MAPK pathway while inhibition of apoptosis is mediated by the PI3K/AKT pathway.

are mediated by PI3K/AKT since reduction in apoptosis was blocked by administration of inhibitors of PI3K or AKT (70,71).

Another action of IGF1 is protection from cellular stress. In particular, IGF1 blocks antidevelopmental effects of heat shock (70,73,74) and menadione (72). There is also a report that IGF1 increased the proportion of frozen-thawed morulae that developed to the blastocyst stage (75). This effect could represent prevention of damage associated with cryopreservation or actions of IGF1 to increase development. IGF1 increases transcript abundance for several genes involved in cellular protection including those involved in regulation of apoptosis and protection against free radicals (74). The cytoprotective effect of IGF1 is probably not the sole result of inhibition of apoptosis. This is so because thermoprotective actions of IGF1 on development were not blocked with a PI3K inhibitor and could not be mimicked by addition of a caspase-3 inhibitor to block apoptosis (70).

Early in development, the embryo is refractory to IGF1. Addition of IGF1 at day 4 after fertilization increased the percent of embryos becoming blastocysts but there was no effect of IGF1 from fertilization to day 4 (69). Similarly, IGF1 protected embryos from heat shock at day 5 of development but not 2-cell embryos (74). Developmental acquisition of responsiveness to IGF1 could reflect changes in IGF1R (62), the need for an active embryonic genome [which occurs at the 8–16 cell stage in the cow (76)], or regulation of key events associated with formation of the morula or blastocyst.

Blastocysts formed in the presence of IGF1 have increased capacity to establish pregnancy when transferred to females (37,77,78). However, as shown in Figure 1, this property has only been shown to occur when recipients were exposed to heat stress. The reason for this interaction between IGF1 treatment and recipient type is not known. It is possible that IGF1-treated embryos are better able to resist maternal heat stress. However, the bovine embryo has gained resistance to maternal heat stress by the blastocyst stage of development (79) and there is little or no seasonal variation in pregnancy rate to embryo transfer (80). It is also not clear what properties of the blastocyst contribute to its increased propensity for posttransfer survival. In some experiments, blastocysts treated with IGF1 had increased cell number (67) and reduced proportion of cells that were apoptotic (66,67), whereas differences in cell number (66,81) and apoptosis (81) were not seen in other experiments.

Administration of somatotropin to embryo transfer recipients also increases embryonic survival (82). However, this action could represent direct actions of somatotropin on embryonic development (65) as well as increased IGF1 in uterine lumen (54,55).

4. Dickkopf 1 and regulation of WNT signaling

The WNT family of regulatory molecules plays important roles in development, oncogenesis, angiogenesis, inflammation, and wound repair (83–86). It is likely that WNTs also regulate the preimplantation embryo. Examination of a microarray database of mRNA from bovine morulae revealed expression of several WNT genes (*WNT1*, *WNT2B*, *WNT3A*, *WNT4*, *WNT5A*, *WNT5B*, *WNT7B*, *WNT8A*, *WNT8B*, *WNT9A*, *WNT9B*, *WNT10A*, *WNT10B*, *WNT11*, and *WNT16*) and FZD receptor genes (*FZD1*, *FZD2*, *FZD3*, *FZD4*, *FZD5*, *FZD6*, *FZD7*, *FZD8*, *FZD9*, and *FZD10*) (87). WNT signaling is complex and characterized by cross-talk between signaling pathways. The most well-characterized signaling cascade is the canonical or β-catenin-dependent pathway. Activation of this pathway requires binding of WNT to FZD and the coreceptor LRP5 or LRP6. A series of downstream events leads to inhibition of proteolysis of β-catenin and its translocation to the nucleus where it interacts with TCF and LEF family transcription factors to regulate gene expression. There are a variety of other signaling cascades activated by WNTs termed noncanonical pathways (83–86). Some of these pathways use FZD as a receptor (planar cell polarity and Ca^{++}-mediated signaling) while other pathways use other receptors such as receptor tyrosine kinase orphan receptor and receptor tyrosine kinase. Often, activations of canonical and noncanonical pathways exert opposite effects on cellular function (84,85).

Overactivation of the canonical WNT signaling pathway can inhibit development of the bovine embryo. Culture of bovine embryos with an agonist of canonical WNT signaling called 2-amino-4-(3,4-(methylenedioxy) benzylamino)-6-(3-methoxyphenyl)pyrimidine (AMBMP) reduced the percentage of embryos that developed to the blastocyst stage and reduced numbers of blastomeres in those blastocysts that did form (87). Cell number was reduced more for TE cells than for cells in the ICM.

WNT signaling in the embryo may be regulated by a secretory inhibitor of canonical WNT signaling called DKK1. Expressed in the bovine endometrium (24,88), DKK1 can bind to LRP5/6 and, in the presence of the transmembrane protein KREMEN, cause its internalization and destruction and thereby prevent formation of the WNT-FZD-LRP5/6 complex (86,89,90). In addition, DKK1 can activate the planar cell polarity pathway (91–93). Addition of DKK1 blocks the inhibitory actions of AMBMP on development (87). In addition, culture of embryos with DKK1 from day 5 to 8 of development increased the percent of cells in the blastocyst labeled as TE, decreased the percent of cells classified as ICM ($CDX2^-$), and increased the percent of cells in the ICM classified as hypoblasts (cells positive for labeling with the

transcription factor GATA6 and negative for labeling with CDX2 and NANOG) (38). These actions of DKK1 are in contrast to the inhibition of TE cells caused by activation of WNT signaling (87).

Based on these experiments, it has been proposed (38) that DKK1 facilitates cell fate decisions in the early embryo, resulting in blastocysts with a higher proportion of embryonic cells committed to the TE fate and of ICM cells differentiating into the hypoblast lineage. These effects of DKK1 likely result from a shift between pluripotency and differentiation, and are not reflective of an increased total cell number because DKK1 does not promote cell proliferation in the early embryo (38).

Like for CSF2 and IGF1, culture of embryos with DKK1 from day 5 to 7 after fertilization improves competence of embryos to establish pregnancy (38). Pregnancy rates at day 32 after ovulation were higher for cows receiving embryos treated with DKK1 than for cows receiving control embryos (Figure 1). The greater pregnancy rate at day 32 for cows receiving embryos treated with DKK1 or CSF2 persisted but differences at calving became nonsignificant (Figure 1).

It is possible that physiological variation in *DKK1* expression in the endometrium contributes to variation in fertility between cows. Expression of *DKK1* is reduced in the endometrium of lactating cows (88) and was lower in inherently subfertile heifers than in more fertile ones (24).

5. Conclusions

Many events must be properly executed for a cleavage-stage embryo to develop to a blastocyst-stage embryo capable of establishing pregnancy. Among these is proliferation of blastomeres, differentiation of TE cells from the outer cells of the morula, formation of the ICM, and differentiation of specific cells in the ICM to hypoblast with retention of pluripotency of the remaining cells of the ICM (now the epiblast). Moreover, the embryo must execute its developmental program while being protected from adverse stimuli in its environment that could cause apoptosis or other cellular damage inducing developmental arrest. These events are regulated by the embryokines that form the focus of this review (Figure 3). In particular, growth of the embryo is enhanced by IGF1, regulation of ICM cell number and capacity for survival is enhanced by

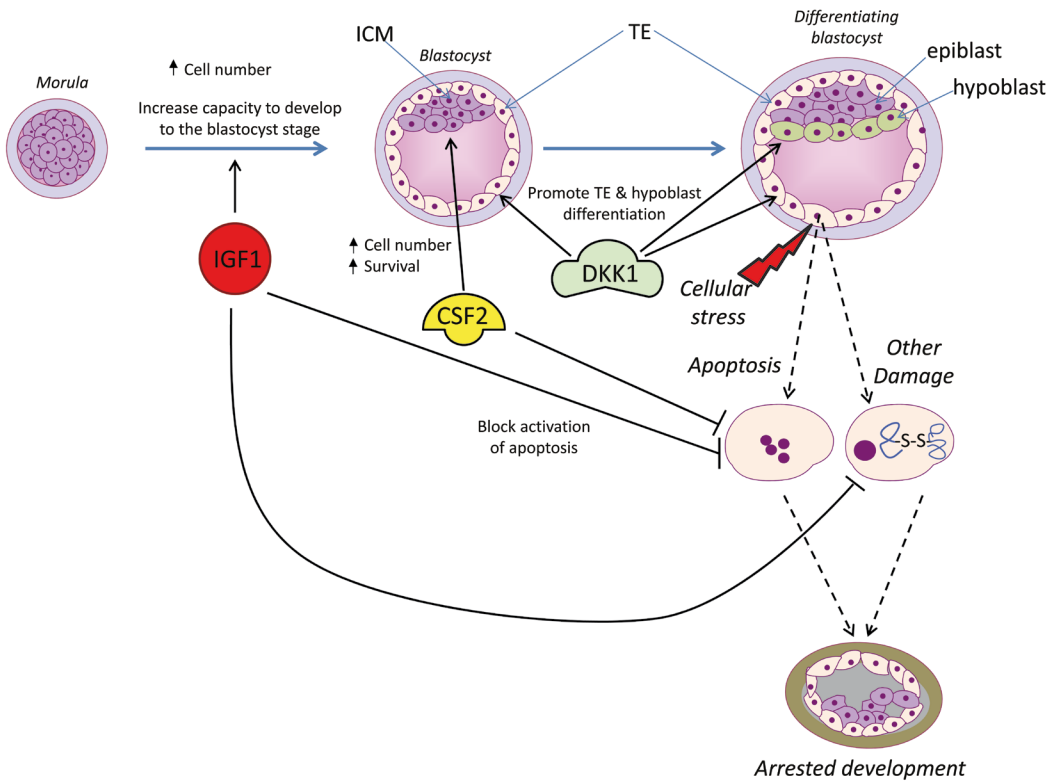

Figure 3. Events in the formation and development of the bovine blastocyst regulated by select embryokines. Development to the blastocyst stage is increased by IGF1, at least in part because it increases cell proliferation. CSF2 affects development of the inner cell mass (ICM) so as to increase its cell number and the ability of isolated ICM to survive in culture. In contrast, DKK1 promotes cell differentiation by promoting development of the trophectoderm (TE) and hypoblast. Both CSF2 and IGF1 protect embryos from stimuli that could otherwise lead to developmental arrest. Both block induction of apoptosis and IGF1 can also reduce apoptosis-independent cellular damage.

CSF2, and differentiation of TE and hypoblast is facilitated by DKK1. Moreover, both CSF2 and IGF1 inhibit induction of apoptosis by proapoptotic stimuli. IGF1 can also reduce apoptosis-independent damage to the embryo, at least that caused by heat shock.

It remains to be determined whether actions of these embryokines are indispensable for optimum development or whether other secretory products of the reproductive tract exert redundant actions. It will also be instructive to determine whether variation between females in secretion of CSF2, IGF1, or DKK1 is an important determinant of fertility and whether manipulation of expression of the genes for these embryokines can improve reproductive function.

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