Developmental disparity between *in vitro*-produced and somatic cell nuclear transfer bovine days 14 and 21 embryos: implications for embryonic loss

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Abstract

In ruminants, the greatest period of embryonic loss coincides with the period of elongation when the embryonic disc is formed and gastrulation occurs prior to implantation. The impact of early embryonic mortality is not only a major obstacle to the cattle breeding industry but also impedes the application of new reproductive technologies such as somatic cell nuclear transfer (SCNT). In the present study, days 14 and 21 bovine embryos, generated by either *in vitro*-production (IVP) or SCNT, performed by either subzonal injection (SUZI) or handmade cloning (HMC), were compared by stereomicroscopy, immunohistochemistry, and transmission electron microscopy to establish *in vivo* developmental milestones. Following morphological examination, samples were characterized for the presence of epiblast (POU5F1), mesoderm (VIM), and neuroectoderm (TUBB3). On D14, only 25, 15, and 7% of IVP, SUZI, and HMC embryos were recovered from the embryos transferred respectively, and similar low recovery rates were noted on D21, suggesting that most of the embryonic loss had already occurred by D14. A number of D14 IVP, SUZI, and HMC embryos lacked an epiblast, but presented trophectoderm and hypoblast. When the epiblast was present, POU5F1 staining was limited to this compartment in all types of embryos. At the ultrastructural level, SCNT embryos displayed abundant secondary lysosomes and vacuoles, had fewer mitochondria, polyribosomes, tight junctions, desmosomes, and tonofilaments than their IVP counterparts. The staining of VIM and TUBB3 was less distinct in SCNT embryos when compared with IVP embryos, indicating slower or compromised development. In conclusion, SCNT and to some degree, IVP embryos displayed a high rate of embryonic mortality before D14 and surviving embryos displayed reduced quality with respect to ultrastructural features and differentiation markers.

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Introduction

The developmental competence of any given embryo is the measurement of a continuum that proceeds throughout its lifecycle. When assisted reproductive technologies (ART) such as somatic cell nuclear transfer (SCNT) are applied, for example in the bovine, there is a need for evaluating embryonic developmental competence as early as at the blastocyst stage in order to select the most competent embryos for transfer. This evaluation is commonly based on blastocyst morphology including the integrity of the embryo, the degree of cellular uniformity, color, and texture as well as the presence of

cellular debris (Lindner & Wright 1983, Stringfellow & Seidel 1998).

However, morphology alone is a poor predictor of subsequent developmental potential, as numerous deviations at the subcellular or molecular level escape stereomicroscopic evaluation. When compared with *in vivo* embryos, *in vitro*-produced (IVP) embryos have smaller embryonic discs (EDs; Bertolini *et al.* 2002) and deviations in volume densities of organelles associated with cellular metabolism (Crosier *et al.* 2001). Moreover, despite similar rates of development at the blastocyst stage, embryos produced in two different culture systems can show profound differences at the level of gene

expression (Wrenzycki *et al.* 2001) as well as in blastomere ultrastructure (Abe *et al.* 1999). IVP embryos also display increased frequencies of chromosomal aberrations (Viuff *et al.* 1999, 2001) and apoptosis (Gjorret *et al.* 2003) when compared with their *in vivo* derived counterparts.

These adverse characteristics are even more pronounced in SCNT embryos (Booth *et al.* 2003, Gjorret *et al.* 2003), which also have an unresolved mitochondrial inheritance pattern (Schatten *et al.* 2005). Moreover, SCNT pregnancies are characterized by placental vascularization deficiencies (Hill *et al.* 2000a), abnormal feto-maternal interactions (Wilmut *et al.* 1986), aberrant gene expression (Daniels *et al.* 2001, Humpherys *et al.* 2002, Wrenzycki *et al.* 2004, Ruddock-D'Cruz *et al.* 2007), or perturbation in the expression of the major histocompatibility complex class I antigens in the placenta of IVP embryos/fetuses (Hill *et al.* 2002). Consequently, first trimester losses of more than 50% of the transferred embryos are common for SCNT pregnancies in cattle, sheep, and goat (Wells *et al.* 1997, 1999, Wilmut *et al.* 1997, Kato *et al.* 1998, Baguisi *et al.* 1999, Hill *et al.* 1999, Heyman *et al.* 2002, Pace *et al.* 2002, Heyman 2005, Tecirlioglu *et al.* 2005).

Indeed, early embryonic mortality has significant economic implications for commercial beef (Diskin *et al.* 2006) and dairy (Moore & Thatcher 2006) breeding programs and is a major impediment to the adoption of a range of ART in the cattle breeding industry. The critical time period of the greatest embryonic loss anticipated for *in vivo* embryos is between days (D) 8 and 16 after fertilization, which coincides with (1) the elongation of the blastocyst involving a significant transition in bovine trophoctoderm development, (2) the formation of the embryonic disc, and (3) the subsequent process of gastrulation by which the germ layers of the embryo are formed (Grealy *et al.* 1996, Dunne *et al.* 2000, Maddox-Hyttel *et al.* 2003, Degrelle *et al.* 2005). In bovine species, blastocyst formation occurs ~7 days after fertilization, and, about 2 weeks later, placentation is initiated with apposition to the uterus (Guillomot 1995). During this time, the trophoblast elongates from 150 µm to 300 mm predominantly from cells that surround the blastocoelic cavity and not from the polar trophoblast cells 'Raubers' layer', which quickly disappears after blastocyst expansion and hatching (Viebahn 1999, Bertolini *et al.* 2002). This process of trophoblast expansion relies on cell multiplication, cell growth, and cell shape remodeling and precedes cellular differentiation (Wintemberger-Torres & Flechon 1974, Betteridge & Flechon 1988). During the initial phase of implantation, important molecular signaling occurs both in the embryo in order to control cell differentiation and between the embryo and the uterus in order to secure placentation and maternal recognition of pregnancy (Spencer *et al.* 2008). Some of the important transcripts related to the latter processes are POU5F1 (van Eijk *et al.* 1999), interferons (IFN- τ ;

Farin & Farin 1995, Roberts *et al.* 1999), prostaglandin H synthases (PTGS1/2; Charpigny *et al.* 1997), protease inhibitors (TKDP family; MacLean *et al.* 2003), pregnancy-associated proteins (PAG1; Green *et al.* 2000), and placental-associated proteins (Ponsuksili *et al.* 2001, Degrelle *et al.* 2005, Hall *et al.* 2005). Progesterone, the hormone of pregnancy, acts on the uterus to stimulate blastocyst preimplantation growth and elongation (Mann & Lamming 2001); however, the specific genes it regulates are only recently being revealed (Spencer *et al.* 2008). It is generally thought that metabolism and protein trafficking occur at the onset of elongation while cellular interactions, cell-to-cell signaling, and cell adhesion occur toward the end of elongation.

Very few studies in cattle, however, have focused on this critical early period (D8–16), the stage that accounts for the vast majority of embryonic loss, in relation to the application of contemporary embryo technologies such as IVP and SCNT. In sheep, it has been documented that serum-enriched embryo culture and SCNT procedures cause a retardation of embryo development and cell differentiation on D7–19 of gestation (Tveden-Nyborg *et al.* 2005). In addition, Bertolini *et al.* (2002) found that on D16 of gestation, the length of bovine IVP embryos was shorter than embryos produced *in vivo* and, moreover, they displayed ED deviations. This study was supported later by similar research findings by Fischer-Brown *et al.* (2005) for D14 IVP embryos where more pregnancies were maintained when there was an ED with no signs of detectable degeneration compared with the ED that was not intact or could not be detected. However, to date a thorough comparative morphological examination, spanning from stereomicroscopy to immunohistochemistry (IHC), employing developmental markers, and transmission electron microscopy (TEM), of bovine IVP versus SCNT embryos at this critical stage of development has not been undertaken.

Consequently, this study compared the development of D14 and D21 bovine embryos, generated by either IVP or SCNT, the latter of which was performed by either traditional subzonal injection (SUZI) or handmade cloning (HMC), to the expected developmental milestones defined for bovine *in vivo* embryos (Maddox-Hyttel *et al.* 2003) using stereomicroscopy, IHC, and TEM. As a baseline, a quantitative assessment of embryo development based on recovery at collection was performed to determine at which point (D14 versus D21) the majority of embryonic loss had occurred.

Results

Recovery rates

Embryo recovery rates on D14 and D21 from IVP, SUZI, and HMC are summarized in Table 1. Briefly, on D14, 6 out of 6 IVP, 3 out of 5 SUZI, and 6 out of 11 HMC recipients yielded embryos. On D21, six out of seven

Table 1 Overall recovery rates and morphological features of D14 and D21 embryos.

Embryo type	Embryos recovered (no. (%))	Embryos presenting the expected cellular compartments ^a (no. (%))
D14 IVP	30/120 (25%)	11/20 (55%)
D14 SUZI	15/102 (15%)	5/11 (45%)
D14 HMC	14/195 (7%)	3/8 (37%)
D21 IVP	31/114 (27%)	NG ^b =8/17 (47%) S ^c =9/17 (53%)
D21 SUZI	13/98 (13%)	NG=4/7 (57%) S=3/7 (43%)
D21 HMC	1/97 (1%)	NG=1/1 (100%)

^aD14: trophoblast, epiblast, and hypoblast; D21: ecto-, meso-, and endoderm. Some of the recovered embryos were processed for molecular studies and, thus, not available for this morphological survey. ^bNG, neural groove stage. ^cS, somite stage.

IVP, five out of five SUZI, and one out of four HMC recipients yielded embryos. On both D14 and D21, there was a marked reduction in the number of recovered embryos in relation to the embryos transferred, particularly in the SUZI and HMC groups.

Mean lengths of embryos

On D14, the mean length of IVP, SUZI, and HMC embryos was 2.5 mm (range 1–5.5 mm), 1.2 mm (range 0.5–5 mm), and 0.8 mm (range 0.4–2 mm) respectively.

On D21, the mean length of IVP, SUZI, and HMC embryos was 3.8 mm (range 1.3–7 mm), 3.2 mm (range 1–5 mm), and 2 mm respectively.

Embryonic cellular compartments

D14 embryos sectioned for IHC or TEM were examined for the presence of epiblast (E), hypoblast (H), and trophectoderm (T) (Fig. 1a and b and Table 1). Similarly, D21 embryos were examined for the presence of the three basic germ layers (ectoderm, mesoderm, and endoderm; Table 1). The D21 embryos ranged from neural groove formation (NG) (Fig. 1c and d) to development of the neural tube (NT) and somites (S) (Fig. 1e and f). No significant differences were detected between IVP, SUZI, and HMC groups in the cellular compartments of the embryo at either D14 or D21.

On D14, a comparison across all groups showed that 35, 65, and 43% of spherical/ovoid, tubular, and filamentous embryos respectively presented a clearly defined epiblast layer.

Qualitative assessment of cellular compartments on D14 by IHC

On D14, a qualitative assessment of the epiblast was performed by IHC staining for the pluripotency marker POU5F1. Six IVP and two HMC embryos chosen for

IHC displayed an epiblast, hypoblast, and trophectoderm layer following hematoxylin and eosin (HE) staining. Of these, all IVP and HMC embryos stained positively for POU5F1. Staining for POU5F1 was exclusively observed in the nuclei of the epiblast cells throughout the embryonic disc in both IVP and HMC embryos (Fig. 2).

Qualitative assessment of cellular compartments on D14 by TEM

Random samples of five IVP, five SUZI, and one HMC embryo with evidence of epiblast, hypoblast, and trophectoderm as determined by HE staining were further analyzed by TEM. For IVP embryos, secondary lysosomes and vacuoles were present in less than one quarter of the epiblast and trophectoderm cells. Scattered signs of apoptosis in the form of chromatin condensation and apoptotic bodies were noted in the epiblast. Some vacuolization and very little apoptosis were seen in the hypoblast. Between the epiblast and the hypoblast, a reticulated basement membrane was seen (Fig. 3b). In one embryo, numerous projections from the hypoblast toward the basement membrane were noted (Fig. 3a). Adjacent trophectoderm cells were linked by prominent tight junctions and conspicuous desmosomes associated with large bundles of tonofilaments (Fig. 3c). Likewise, adjacent epiblast cells were linked by tight junctions and the peripheral epiblast cells of the embryonic disc and the adjacent trophectoderm cells were also linked by such structures. The epiblast cells of IVP embryos presented abundant polyribosomes and mature mitochondria (Fig. 3d). In some IVP, SUZI, and HMC embryos, persistence of portions of the Rauber's layer, i.e., the trophectoderm initially covering the epiblast, was noted. In such embryos, the epiblast cells beneath the degrading Rauber's layer presented a higher number of secondary lysosomes and vacuoles (Fig. 3e).

In general, SUZI and HMC embryos presented less conspicuous tight junctions and desmosomes between adjacent epiblast cells as well as between epiblast cells and adjacent trophectoderm cells (Fig. 4a). Likewise, the tonofilaments were less abundant. SUZI and HMC embryos presented larger numbers of vacuoles, secondary lysosomes, and apoptotic features (chromatin condensation, formation of apoptotic bodies, phagocytosis of apoptotic bodies), which were present in about one-third to one-half of epiblast cells (Fig. 4b and d) and in about two-thirds of trophectoderm cells. The mitochondria, in general, presented less cristae. Hypoblast cells were also affected to a large extent, particularly under the epiblast. In one out of five SUZI and one out of one HMC embryos, the basement membrane between the epiblast and the hypoblast was disintegrated (Fig. 4c).

Figure 1 (a) D14 spherical SUZI embryo as seen under a stereomicroscope. (b) Light micrograph of embryo in (a) showing the epiblast (E), hypoblast (H), and trophoblast (T). (c) D21 NG-stage IVP embryo (neural groove indicated by arrowheads) as seen under a stereomicroscope. (d) Light micrograph of embryo in (c) showing the neural groove (NG), neuroectoderm (NE), mesoderm (M), amnion (A), and endoderm (E). (e) D21 S-stage IVP embryo with somites (arrows) as seen under a stereomicroscope. (f) Light micrograph of embryo in (e) showing amnion (A), surface ectoderm (SE), neural tube (NT), somites (S), notochord (N), intermediate mesoderm (IM), splanchnic mesoderm (SPM), somatic mesoderm (SM), endoderm (E), and hypoblast (H).

Qualitative assessment of cellular compartments on D21 by IHC

On D21, the recovered embryos were classified as having a NG or a partial NT combined with the presence of somites. The distribution of these developmental stages did not vary between the IVP, SUZI, and HMC embryos. Likewise, the proportions of embryos displaying the expected cellular compartments, i.e., ectoderm, endoderm, and mesoderm, were not different.

Figure 2 Epiblast cells (E) from a D14 IVP embryo showing nuclear staining for POU5F1. As expected, there was no staining in the hypoblast (H) or trophoblast (T).

At the NG stage, IVP embryos displayed strong staining for VIM in all mesoderm compartments (Fig. 5a), and staining for TUBB3 was found in the neural ectoderm as well and to a lesser extent in the extra-embryonic mesoderm (Fig. 5b and Table 2). SCNT embryos, on the other hand, displayed diminished or absence of VIM staining of the mesoderm (Fig. 5c). This pattern of weaker staining in SCNT versus IVP embryos was also seen for TUBB3 staining at the NG stage (Fig. 5d).

At the somite stage, the IVP embryos still displayed VIM staining in mesodermal compartments including the somites (Fig. 6a) whereas TUBB3 staining predominantly was noted in the NT (Fig. 6b). Again, staining of VIM (Fig. 6c) and TUBB3 (Fig. 6d) was either diminished or absent in SCNT (only SUZI recovered) when compared with IVP embryos.

Discussion

The present study examined D14 and D21 IVP and SCNT (SUZI and HMC) bovine embryos by stereomicroscopy, IHC, and TEM. Two nuclear transfer techniques were employed, SUZI and HMC, to allow the production of sufficient numbers of cloned blastocysts prior to embryo transfer. In a previous study, pregnancy outcome from SUZI and HMC procedures using the same donor cell did not differ (Tecirlioglu *et al.* 2005). A quantitative

Figure 3 D14 IVP embryo. (a) Light micrograph showing the epiblast (E), hypoblast (H), trophoblast (T), and remnants of Rauber's layer (RL). (b) Basement membrane (BM) between the hypoblast (H) and the epiblast (E). (c) Tight junctions (TJ) and desmosomes (D) between epiblast cells and tonofilaments (TF) associated with the desmosomes. (d) Mature mitochondria (M) and poly-ribosomes (PR) in the cytoplasm of an epiblast cell delineated by the basement membrane (BM) toward the hypoblast. (e) Extensive degeneration of epiblast cells (E) presenting large secondary lysosomes (L) under Rauber's layer (RL) consisting of degenerated electron-dense trophoblast cells characterized by lysosomes and vacuoles (V).

Figure 4 (a) Well-defined tight junctions (TJ) and desmosomes (D) between neighboring trophoblast cells (T) with a high number of vesicles (V) in a D14 HMC embryo. (b) Epiblast cells with a large number of secondary lysosomes (L) and vacuoles (V) in a D14 SUZ1 embryo. (c) Degrading basement membrane (BM) between the epiblast (E) and hypoblast (H) in a D14 SUZ1 embryo. (d) Apoptotic bodies (AB) between epiblast cells (E1 and E2) in a D14 SUZ1 embryo.

Figure 5 (a and b) D21 NG-stage IVP embryo. (a) Intense VIM staining of intra-embryonic mesoderm (M) and extra-embryonic mesoderm (EEM). (b) Homogeneous and intense staining of TUBB3 through the neuroectoderm (NE) and to some degree in the paraxial mesoderm (M). (c and d) D21 NG-stage HMC embryo. (c) Lack of VIM staining in the intra-embryonic mesoderm (M) and extra-embryonic mesoderm (EEM). (d) Weaker and less homogeneous staining of TUBB3 in the neuroectoderm (NE) and the intra-embryonic mesoderm (M).

assessment of embryo development based on recovery at collection was used as a baseline to assess expected developmental milestones defined for bovine *in vivo* embryos.

The 2-week period following transfer was associated with significant embryonic loss in all groups of embryos. Thus, 75, 85, and 93% of IVP, SUZI, and HMC embryos respectively were lost as evaluated by the flushing data on D14, and almost the same ratios of loss were noted on D21. These low figures probably represent a combination of high embryo mortality and technical problems in retrieving the D14 embryos. Embryo collection on D14 presented a very diverse range of materials from the uterine cavity including clearly distinguishable embryos mixed with trophectoderm vesicles and cellular debris. The use of vitrification to produce large numbers of embryos for transfer may have attributed to some of the embryonic loss, although this procedure has previously been used extensively to generate cloned calves (Tecirlioglu *et al.* 2005). In the study by Tecirlioglu *et al.* (2005), pregnancy outcome

with fresh and vitrified cloned embryos generated with the same donor cell did not differ. All embryos vitrified were thawed prior to embryo transfer. Following recovery, only embryos resembling grade 1 were selected for transfer. Vitrified blastocysts were morphologically equivalent to the fresh embryos transferred at the time of embryo transfer. Previous studies on artificially inseminated cows have demonstrated an incidence of embryonic loss between D8 and D18 of 30–40% (Ayalon 1978, Diskin & Sreenan 1980, Roche *et al.* 1981, Dunne *et al.* 2000). With SCNT, a significantly higher incidence of embryonic loss exceeding 50% by D24 has been reported (Farin & Farin 1995, Hill *et al.* 2000b, Peterson & Lee 2003).

The development of both IVP and SCNT embryos was delayed and even in some cases abnormal when compared with the expected developmental milestones defined for *in vivo* embryonic development. SCNT embryos on both D14 and D21 were developmentally delayed (i.e., shorter in length) when compared with equivalent IVP embryos. Moreover, measurements

Table 2 Localization and semi-quantitative intensity of VIM (V) and TUBB3 (T) staining in bovine D21 embryos at the neural groove (NG) or somite (S) stage produced either by *in vitro*-produced (IVP) or somatic cell nuclear transfer (SCNT) (subzonal injection, SUZI; handmade cloning, HMC).

Embryo type	Extra-embryonic mesoderm	Embryonic mesoderm	Neural groove	Somites	Neural tube
IVP	V++	V+++	V++	N/A	N/A
NG stage	T++	T+++	T+++		
SUZI	V++	V+	V–	N/A	N/A
NG stage	T++	T++	T++		
HMC	V+	V+	V–	N/A	N/A
NG stage	T+	T+	T+		
IVP	V+++	V+++	N/A	V+++	V++
S stage	T++	T+++		T++	T+++
SUZI	V++	V++	N/A	V++	V++
S stage	T+	T–		T++	T++

–, absent; +, weak; ++, strong staining in some cells; +++, strong staining in all cells; N/A, staining not expected in specified germ layer as it did not match developmental stage.

Figure 6 (a and b) D21 S-stage IVP embryo. (a) VIM staining of the neural ectoderm (NE), somite mesoderm (M), and extra-embryonic mesoderm (EEM). (b) Uniform staining of TUBB3 in the neuroectoderm (NE) and mesoderm (M). (c and d) D21 S-stage SUZI embryo. (c) Weaker intensity of VIM staining in the neuroectoderm (NE), mesoderm (M), and extra-embryonic mesoderm (EEM). (d) Less intense staining of TUBB3 in the neuroectoderm (NE) and somite mesoderm (M).

within the SCNT group detected a more pronounced delay in development in the HMC group. On D14, the lack of a clearly defined epiblast at the stereomicroscopic level was a common finding. In many cases, when such embryos were further processed for IHC or TEM, a well-developed trophoblast and hypoblast were revealed, but the epiblast was lacking. As this phenomenon was equally common in spherical/ovoid embryos and the more advanced filamentous embryos, it seems as if embryonic growth, at least for a period, is not disturbed by the lack of an epiblast. Similar findings have been reported for ovine SCNT embryos (Tveden-Nyborg *et al.* 2005). It may be speculated that hypoblast formation is a dominant process, which, if the inner cell mass is low in cell number, consumes all cells of the inner cell mass. However, it was noticed that tubular embryos were most likely to possess an epiblast. This corresponds with the previous studies where successful elongated embryos provided the greatest chance to establish and maintain pregnancy (Wilson *et al.* 2000, Fischer-Brown *et al.* 2005).

All D14 embryos with an epiblast, as previously identified by HE staining, showed an expression of POU5F1 irrespective of whether they were of IVP or SCNT origin. This observation is in line with the previous studies of D14 *in vivo* bovine (van Eijk *et al.* 1999, Vejlsted *et al.* 2005) and *in vivo* porcine embryos (Vejlsted *et al.* 2006b) and is a further confirmation of embryo viability.

At the ultrastructural level, both IVP and SCNT embryos were compared with *in vivo* embryo development as previously described by Maddox-Hyttel *et al.* (2003) to determine the developmental deviations in individual cellular compartments. Both D14 IVP and SCNT embryos displayed epiblast and hypoblast cell

layers, which in many respects, were similar to those previously described for bovine *in vivo* embryos (Maddox-Hyttel *et al.* 2003) although differences between IVP and SCNT embryos were recorded. The hypoblast cells of IVP embryos showed less apoptosis and fewer vacuoles as well as more mitochondria and polyribosomes when compared with SCNT embryos. The hypoblast cells form a closed compartment referred to as the primitive yolk sac, most of which remains outside the embryo forming the extra-embryonic yolk sac (Noden & de Lahunta 1985). The hypoblast may possess important functions in inducing particular differentiation patterns in the overlying epiblast. Hence, in the rabbit embryonic disc, an 'anterior marginal crescent' (AMC), in which a thickening of the hypoblast and the overlying epiblast is seen, has been reported (Viebahn *et al.* 1995). The thickened hypoblast is believed to possess neuronal-inducing properties. A similar phenomenon is reported in the mouse in the form of the so-called anterior visceral endoderm (AVE; (Thomas & Beddington 1996, Knoetgen *et al.* 1999, 2000, Kimura *et al.* 2000, Perea-Gomez *et al.* 2001, 2002, Idkowiak *et al.* 2004). It has also been found that during hypoblast formation, epiblast cells generate stellate cell-like projections in their basal aspect, thus providing a meshwork for the advancing hypoblast cells (Watt *et al.* 1993). Therefore, any disruption of hypoblast function may have consequences for further development.

Between the hypoblast and epiblast, a basement membrane that may have a role in embryo polarity exists (Yurchenco & Schittny 1990). This membrane was intact and well formed in IVP embryos but appeared disintegrated in SCNT embryos. Tight junctions between trophoblast (TE) cells were found in all embryos but were not as well defined in the SCNT embryos.

Desmosomes were abundant in both IVP and SCNT embryos, although more dense clusters of tonofilaments, important in accommodating stretching of cells as they change in size and shape, were found in greater abundance in IVP than SCNT embryos. A reduction in the numbers of these cytoskeletal structures could lead to structural damage as cells grow, or indicate premature ageing of cells (Cross *et al.* 1994).

Polyribosomes and mature mitochondria were also less abundant in SCNT embryos when compared with IVP embryos. The mitochondria in the SCNT embryos appeared immature having less cristae, a phenomenon that has been associated with a decreased ability to metabolize lipid (Crosier *et al.* 2000). Polyribosomes are necessary for growth by providing a continuous supply of structural proteins (Carnegie *et al.* 1985).

Although secondary lysosomes and vacuoles were observed occasionally in IVP embryos, up to two-thirds of TE cells and up to half of the epiblast cells in SCNT embryos displayed these features. The secondary lysosomes and vacuoles frequently contained cellular debris resulting from autophagocytosis or ingestion of embryonic cell fragments and indicated a high level of cellular loss in the embryos. Secondary lysosomes were particularly evident in epiblast cells that still were covered by remnants of Rauber's layer indicating an active role of the epiblasts in the removal of the trophoctoderm covering at this time of development. Furthermore, communication between the embryonic disc and the extra-embryonic tissues could have significant importance for blastocyst viability since differentiation during gastrulation establishes the primary germ layers and the embryonic developmental axes prior to implantation (Hue *et al.* 2007).

Embryos obtained on D21 of development would be expected to have developed the three germ layers and to have begun the process of neurulation. Two markers were chosen for this study to determine developmental normality at this stage, VIM (a mesoderm marker) and TUBB3 (a marker of neuroectoderm). VIM is first expressed in the epiblast (Maddox-Hyttel *et al.* 2003). Later, it persists in mesoderm-derived tissues such as the mesenchyme, somites, and developing heart as well as in mesenchymal derivatives of the neural crest, and in the NG and tube (Jackson *et al.* 1981). IVP embryos displayed the expected localization of VIM to mesenchyme, somites, and NG and tube. SCNT embryos, however, displayed weaker staining in mesoderm components, including the somites, and the NT. The absence or diminished staining in these tissues could be an indicator of developmental abnormalities that may manifest themselves later in development. SCNT cattle embryos have been reported to show a higher body weight, right ventricular enlargement, kidney abnormalities, and limb deformities (Rhind *et al.* 2003), as well as contracted flexor tendons (Panarace *et al.* 2007).

Staining of TUBB3 in the anterior ectoderm has been shown to correspond with the onset of neural differentiation at the NG stage. Later, it also localizes to the NT (Vejlsted *et al.* 2006a). Again, the IVP embryos displayed the expected staining for TUBB3, while the intensity of this staining was much lower in SCNT embryos. These findings suggest aberrations in the specification of the neural ectoderm. A common mortality factor in 15% of SCNT calves before weaning is, among other things, neurological abnormalities (Renard *et al.* 2002, Wells 2005).

In conclusion, SCNT and, to some degree, IVP embryos displayed the expected high rate of embryonic mortality before D14. Furthermore, while SCNT embryos surviving to D14 displayed the expected cellular compartments (epiblast, hypoblast, and trophoctoderm) at a similar rate as their IVP counterparts, ultrastructural features predicted a lower embryonic viability when compared with IVP embryos. Likewise, on D21 the SCNT embryos displayed the expected cellular compartments (ectoderm, mesoderm, and endoderm) at the same rate as their IVP counterparts. However, their expression of differentiation markers was less pronounced than that in IVP embryos, again suggesting reduced viability. Combining these observations gives further insight into the developmental irregularities between IVP and SCNT embryos on D14 and 21 and suggests the possible mechanisms by which many abnormalities are established following IVP and SCNT.

Materials and Methods

Animal ethics

All experiments were approved by the Monash University Animal Experimentation Ethics Committee, which adheres to the Victorian Prevention of Cruelty to Animals Act Regulation 1986 and the NHMRC Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (6th ed., 1997).

General aspects

All Chemicals used for the production of IVP and NT embryos were purchased from Sigma Chemical Co. unless otherwise specified. All procedures were performed in four-well Nunc dishes (Nunc, Roskilde, Denmark) at 39 °C, except for the process of electrical fusion, which was performed at room temperature.

Bovine oocyte collection and in vitro maturation

Bovine ovaries collected from a local slaughterhouse were transported within 2 h at 30–35 °C to the laboratory. Ovarian antral follicles (2–8 mm) were aspirated using an 18 gage needle, and cumulus–oocyte complexes (COCs) were collected into TCM-199 (Gibco, BRL/Life Technologies) supplemented with 25 mM HEPES (TCM-H) and 30 IU/ml heparin (Pharmacia & Upjohn Pty. Ltd) and 2% β -irradiated fetal calf serum

(FCS; Lot No. IL0403; JRH Biosciences Pty., Ltd, Melbourne, Victoria, Australia). COCs exhibiting a homogeneous cytoplasm and surrounded by at least three layers of compact cumulus cells were selected and matured in groups of 50 in 600 µl of TCM-199 supplemented with 5 µg/ml LH (Lutropin-V; Bioniche Animal Health, A/Asia Pty. Ltd, Armidale, Victoria, Australia), 1 µg/ml β -estradiol, 100 IU/100 µg/ml penicillin/streptomycin, and 10% FCS at 39 °C in a humidified atmosphere of 5% CO₂ in air for 18–22 h.

Production of in vitro-produced (IVP) blastocysts

Frozen/thawed spermatozoa from bulls of proven fertility were used for all *in vitro* fertilization procedures. Presumptive metaphase II oocytes following *in vitro* maturation were co-cultured with spermatozoa (1×10^6 spermatozoa/ml) in fertilization medium supplemented with heparin (0.5 mg/ml), hypotaurine (1.65 µg/ml), epinephrine (0.27 µg/ml), and penicillamine (4.5 µg/ml) for 24 h at 39 °C in 5% CO₂ in humidified air as described previously (Parrish *et al.* 1988). Following 24 h of spermatozoa–oocyte co-incubation, cumulus cells were removed from presumptive zygotes by vortexing for 90 s. Denuded embryos were washed in TCM-H supplemented with 5% FCS before being transferred into four-well Nunc plates (Nunc) containing 500 µl Bovine Vitro Cleave (Cook Australia, Brisbane, QLD, Australia) medium for 5 days at 39 °C in a humidified atmosphere of 5% O₂, 5% CO₂, and 90% N₂. On D5, embryos were transferred into Bovine Vitro Blast (Cook Australia) medium supplemented with 4% charcoal-treated bovine serum, 4% supernatant obtained from mouse embryonic carcinoma cells containing fibroblast growth factor-4 (FGF4), and 20 µg/ml heparin. The cleavage rate (i.e., fertilization rate) and the proportion of blastocysts were determined on D2 (48-h post-insemination, hpi; D0 = day of fertilization) and D7 (168 hpi) respectively.

Grade 1 blastocysts from all treatments groups were selected for transfer on D7 according to International Embryo Transfer Society Guidelines.

Preparation of donor cells for nuclear transfer

A fibroblast donor cell line was isolated from a skin sample taken from an adult Holstein–Friesian bull with elite genetics and verified for a normal karyotype (87%; $n=60$). Single actively dividing donor cells were prepared on the day of SCNT using trypsin/EDTA and resuspended in 1 ml TCM-H supplemented with 20% FCS and 4 mg/ml BSA (Immuno-Chemical Products, Auckland, New Zealand), and left at room temperature (3–4 h) until required for fusion.

Production of NT bovine embryos by HMC and SUZI

Construction of HMC embryos was performed according to previously described methods (Tecirlioglu *et al.* 2004). Briefly, zona pellucidae were removed following a brief incubation in protease (5 mg/ml) and zona-free cytoplasts were selected following manual bisection under a stereomicroscope using an embryo-splitting blade (AB Technology, Pullman, WA,

Australia) and DNA staining with Hoechst 33342 (20 µg/ml) under epifluorescence to discard karyoplasts.

Reconstruction of cytoplast and donor cell was aided with 200 µg/ml phytohemagglutinin (Tecirlioglu *et al.* 2005). Following attachment, cytoplast–cell couplets and singles cytoplasts were equilibrated in fusion medium (0.3 M mannitol, 0.1 mM MgSO₄, 0.05 mM CaCl₂) before being transferred to the fusion chamber with a 0.5 mm gap between the wires (microslide 450; Cat. No. 01-000209-01, BTX Ltd, Holliston, MA, USA) containing fusion medium. Cytoplast–fibroblast–cytoplast triplets were fused using a single electrical pulse (3.78 kV/cm for 4 µs).

Preparation of SUZI cloned embryos were produced as previously reported (Daniels *et al.* 2001), except that cell fusion used two electrical pulses (1.7 kV/cm for 15 µs $\times$ 2 pulses administered 0.1 s apart). All embryos were examined morphologically after 20 min and non-fused embryos were fused again. Embryos that were not fused following this second fusion were discarded.

Parthenogenic activation

Cloned (HMC and SCNT) embryos were chemically activated 4 h after fusion with calcium ionophore A-23187 (5 µM, 4 min) in TCM-H, then washed thrice in TCM-H supplemented with 20% FCS before being transferred to Bovine Vitro Cleave (Cook Australia) medium supplemented with 2 mM 6-dimethylaminopurine (6-DMAP) for a further 3-h incubation in 5% CO₂ in air.

In vitro embryo culture

Reconstructed (HMC and SUZI) cloned embryos were washed with Bovine Vitro Cleave, before culture of HMC embryos in a single well-of-the-well (WOW system) as described by Vajta *et al.* (2000) or in groups for SUZI embryos in four-well Nunc plates containing culture medium (Bovine Vitro Cleave and Blast; Cook Australia) as described for IVP embryos.

Embryo vitrification and in-straw thawing

Blastocysts were vitrified using the open-pulled straw (OPS) method as described by Vajta *et al.* (1998). Embryos were thawed using the in-straw dilution method in 0.25 ml transfer straws containing TCM-H supplemented with 0.2 M sucrose and 40% FCS prior to embryo transfer as previously described (Vajta *et al.* 1999, Tecirlioglu *et al.* 2005).

Embryo transfer and collection

Holstein–Friesian recipient cows were synchronized with an intra-vaginal progesterone-releasing device inserted for 7 days (CIDR; InterAg, Hamilton, New Zealand). The heifers received an i.m. injection of synthetic prostaglandin (Lutalyse; Pharmacia) 24 h before CIDR removal. Embryos were transferred on D7 to recipient donors.

For embryo collection on D14, 120 IVP embryos (80 fresh and 40 vitrified) were transferred into 6 recipients, 102 SUZI

embryos (56 fresh and 46 vitrified) into 5 recipients, and 195 HMC embryos (65 fresh and 148 vitrified) into 11 recipients.

For collection on D21, 114 IVP embryos (51 fresh and 83 vitrified) were transferred into seven recipients, 98 SUZI embryos (45 fresh and 53 vitrified) into five recipients and 97 HMC embryos (6 fresh and 91 vitrified) into four recipients. In total, 726 embryos were transferred into 38 recipients with an average of 19 embryos per recipient.

On D14 and D21, recipients were slaughtered and their reproductive tracts transported to the laboratory. Embryos were recovered using a non-surgical equine embryo-flushing catheter (Pacific Vet, Braeside, VIC, Australia) inserted through a small incision made in the base of each uterine horn fitted with an embryo collection filter (0.2 µm; Pacific Vet). Each uterine lumen was flushed at least twice with 100 ml PBS (Oxoid Ltd, Basingstoke, Hampshire, England), supplemented with 5% FCS (Gibco BRL, Life Technologies). Gentle massage was employed to each uterine horn and the uterine body to recover flushing medium containing embryos. Approximately 60 ml fluid containing potential embryos were transferred to 10 cm Falcon tissue culture dishes (Becton-Dickinson, North Ride, NSW, Australia), to allow searching for embryos under a light microscope (Olympus Optical Co., Tokyo, Japan). Individual embryos were collected in fresh PBS/FCS solution. Upon completion, embryos were randomly allocated for further processing either for molecular analysis, IHC, or TEM.

After fixation (see later), all embryos were photographed on an Olympus Bx 51 microscope and images manipulated with Scion Image software (Apple MacIntosh, USA).

Processing of embryos for IHC

Embryos (D14: 16 IVP, 4 SUZI, and 3 HMC and D21: 11 IVP, 4 SUZI, and 1 HMC) were placed singly in four-well dishes containing pre-chilled 4% paraformaldehyde (PF; Sigma) in PBS (pH 7.2) for 1 h and then rinsed with fresh PBS. Embryos were left in PBS at 4 °C overnight before embedding in blocks of 4% agar to allow processing to wax in a Leica TP 1020 processor (Histocassette; Leica Instruments GmbH, Wetzlar, Germany). After processing, embryos were cut into 5 µm sections on a Leica RM 2135 microtome (Leica Instruments) using a steel knife (Feather Safety Razor Co., Ltd, Osaka, Japan) and placed on Superfrost Plus slides (Menzel-Glässer GmbH, Strassburg, Germany). These slides were left in a 37 °C oven overnight.

One in every 10 slides were stained with HE to visualize gross morphology and to determine which slides to select for the IHC staining. For IHC characterization, selected sections adjacent to either epiblast or displaying the three germ layers were dewaxed and rehydrated. Endogenous peroxidase activity was blocked with 3% (v/v) H₂O₂ in ethanol for 10 min before antigen retrieval by microwave treatment in 0.01 M sodium citrate buffer (pH 6) for 3×5 min (Shi *et al.* 1991). Non-specific binding of antibodies was blocked with a ready-to-use, serum-free, protein blocking solution (X0909; DakoCytomation, Glostrup, Denmark). Subsequently, the sections were pre-blocked in protein blocking solution (X0909; DakoCytomation). Sections were then exposed to one of the following primary antibodies; mouse monoclonal

anti-human VIM (0.5 mg/ml; 1:500; 18-0052, Zymed Laboratories, Inc., San Francisco, CA, USA) at 4 °C overnight, rabbit polyclonal anti-human POU class 5 homeobox 1 (POU5F1; 200 µg/ml; 1:250; SC-8628, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), and mouse monoclonal anti-TUBB3 (3.1 mg/ml; 1:1000; T8660) for 1 h at room temperature. The site of antigen-antibody reaction of POU5F1 and VIM was revealed with a triple-layer method using biotinylated Ig and peroxidase-labeled streptavidin (DakoCytomation, K0690), followed by development in 3-amino-9-ethylcarbazole (AEC)-H₂O₂ (Zymed Laboratories, Inc.) as per the manufacturer's instructions for 5 min at room temperature. Sections were counterstained with Mayer's hematoxylin (Sigma Diagnostics) and then mounted in Faramount aqueous mounting medium (S3025, DakoCytomation). The site of antigen-antibody reaction of TUBB3 was revealed with goat anti-rabbit Ig antibodies labeled with Alexa 488 (diluted 1:400; Molecular Probes Europe BV, Leiden, The Netherlands) and the sections were counterstained with bisbenzimidazole. For negative controls, the antibodies to POU5F1 were preabsorbed with the corresponding peptide antigen (Santa Cruz Biotechnology; SC-4420 WB) for 2 h at 4 °C, while the antibodies for VIM and TUBB3 were interchanged with identical concentrations of the corresponding Ig: IgG1 (DakoCytomation, X0931) and IgG2a (DakoCytomation, X0943) respectively. Light microscopy slides were observed on a Leica microscope and images captured and manipulated using Paint Shop Pro 7.0 software. Fluorescent images were acquired in a Leica DMRB microscope equipped with selective epi-illumination filter blocks for AMCA/bisbenzimidazole excitation (A), FITC excitation (L4), and Texas red excitation (TX) and a COHU CCD camera controlled by a Leica Q-FISH system.

Processing of embryos for TEM

Embryos (D14; four IVP, seven SUZI, and five HMC) were fixed for 1 h in 3% glutaraldehyde in 0.1 M cacodylate buffer (ProSciTech, Thuringowa, QLD, Australia). Specimens were embedded in 4% agar (Bacto-agar; Difco Laboratories, Detroit, MI, USA) under stereomicroscopy and post-fixed in 4% OsO₄ in the same buffer for 1 h at 4 °C. Embryos were then block stained with aqueous 0.5% uranyl acetate overnight, dehydrated through ascending concentrations of ethanol, transferred to propylene oxide, and embedded in Epon 812. Sections were cut serially (2 µm) using a glass knife. Selected sections were re-embedded in Epon 812 (Hyttel & Madsen 1987), cut into 70 nm thick sections, counterstained with uranyl acetate and lead citrate, collected on copper grids, and examined on a transmission electron microscope (CM 100; Phillips, Darmstadt, The Netherlands) before recording images digitally.

Statistical analyses

The proportional data for the recovery of embryos were analyzed by the χ^2 -test. Unless stated otherwise, significance was declared at $P < 0.05$.

Declaration of interest

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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