

# Inhibition of trophoblast stem cell potential in chorionic ectoderm coincides with occlusion of the ectoplacental cavity in the mouse

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## SUMMARY

At the blastocyst stage of pre-implantation mouse development, close contact of polar trophoderm with the inner cell mass (ICM) promotes proliferation of undifferentiated diploid trophoblast. However, ICM/polar trophoderm intimacy is not maintained during post-implantation development, raising the question of how growth of undifferentiated trophoblast is controlled during this time. The search for the cellular basis of trophoblast proliferation in post-implantation development was addressed with an *in vitro* spatial and temporal analysis of fibroblast growth factor 4-dependent trophoblast stem cell potential. Two post-implantation derivatives of the polar trophoderm – early-streak extra-embryonic ectoderm and late-streak chorionic ectoderm – were microdissected into fractions along their proximodistal axis and thoroughly dissociated for trophoblast stem cell culture. Results indicated that cells with trophoblast stem cell potential were distributed throughout the extra-embryonic/chorionic ectoderm, an observation that is probably attributable to non-coherent growth patterns exhibited by single extra-embryonic ectoderm cells at the

onset of gastrulation. Furthermore, the frequency of cells with trophoblast stem cell potential increased steadily in extra-embryonic/chorionic ectoderm until the first somite pairs formed, decreasing thereafter in a manner independent of proximity to the allantois. Coincident with occlusion of the ectoplacental cavity via union between chorionic ectoderm and the ectoplacental cone, a decline in the frequency of mitotic chorionic ectoderm cells *in vivo*, and of trophoblast stem cell potential *in vitro*, was observed. These findings suggest that the ectoplacental cavity may participate in maintaining proliferation throughout the developing chorionic ectoderm and, thus, in supporting its stem cell potential. Together with previous observations, we discuss the possibility that fluid-filled cavities may play a general role in the development of tissues that border them.

Key words: Trophoblast stem cell, FGF4, Extra-embryonic ectoderm, Chorionic ectoderm, Ectoplacental cavity, Chorioallantoic placenta, Mouse, Embryo, Epithelium

## INTRODUCTION

In the mouse, the first cells to differentiate from the fertilised egg are those of the extra-embryonic trophoblast lineage, whose ultimate role is to facilitate maternal/foetal exchange in the mature chorioallantoic placenta (Cross, 2000). In pre-implantation development, trophoblast cells are derived from trophoderm, the external epithelial layer of the blastocyst. In the blastocyst, proliferation is limited to the polar trophoderm (pTE) that overlies the inner cell mass (ICM) (Gardner and Johnson, 1972; Gardner et al., 1973), while cells of the mural trophoderm (mTE), which lack ICM contact, rapidly form terminally differentiated postmitotic trophoblast ‘giant’ cells (TGCs) through multiplication of the entire genome via repeated rounds of endoreduplication (Barlow and Sherman, 1972; Barlow and Sherman, 1974). Thus, the pTE retains an undifferentiated diploid state while TGCs become polyploid (Bower, 1987; Varmuza et al., 1988).

Multi-potency of the pTE (Gardner et al., 1973) and uni-

directionality of the terminal differentiation of its derivatives (Johnson and Rossant, 1981; Carney et al., 1993) underpin expansion of the trophoblast lineage in mouse development. Central to this issue during post-implantation development is the mechanism of growth control of the immediate derivative of the pTE, the extra-embryonic ectoderm (ExE). That trophoblasts present in the chorioallantoic placenta are believed to originate exclusively from the pTE (Gardner et al., 1973; Copp, 1978; Dyce et al., 1987; Gardner, 1996) suggests that cells derived from this trophodermal sub-region retain an extensive capacity for self-renewal. This has been confirmed *in vitro* with the isolation and characterisation of multi-potent fibroblast growth factor 4 (FGF4)-dependent trophoblast stem cells (TSCs) derived from dissociated early-streak [~6.5 dpc (days post coitum)] ExEs (Tanaka et al., 1998). Therefore, progenitor cells which can give rise to TSCs *in vitro*, appear to exist until at least the onset of gastrulation.

Hence, TSC progenitors are vitally important in elucidating mechanisms responsible for the expansion of the

undifferentiated diploid trophoblast population during development. At the molecular level, the derivation of FGF4-dependent TSCs corroborates other studies that implicate *Fgf4* (Niswander and Martin, 1992; Rappolee et al., 1994; Feldman et al., 1995; Brison and Schultz, 1996) and its candidate receptor, *Fgfr2* (Arman et al., 1998; Haffner-Krausz et al., 1999), as components of the ICM-derived signal mediating pTE growth. However, it is not currently known whether TSC progenitors are retained in the chorionic ectoderm (ChE), the derivative of the ExE, as late as 8.5 dpc, although this trophoblast derivative still appears to be morphologically undifferentiated and contains diploid mitotic cells (Barlow and Sherman, 1972; Rossant and Ofer, 1977). Until the spatial and temporal distribution of these mitotic cells is better understood, a complete portrait of the mechanisms responsible for extensive proliferation in the ExE/ChE, especially its TSC population, will remain elusive.

Although experimental manipulation of blastocysts has demonstrated that the ICM, the precursor tissue of the embryo proper, maintains mitoses in pTE by virtue of close contact (Gardner and Johnson, 1972; Gardner et al., 1973; Ansell and Snow, 1975; Goldstein et al., 1975), it is presently unknown whether expansion of the ExE is also mediated by close contact with the post-implantation derivative of the ICM, the epiblast. Tanaka et al. (Tanaka et al., 1998) proposed that in the early-streak ExE, TSC progenitors may be limited to a discrete stratum abutting the *Fgf4*-expressing epiblast (Niswander and Martin, 1992), owing to the presumed necessity of close contact for the reception of an FGF4 signal.

This hypothesis is questionable, as two observations on the growth of the nascent conceptus appear inconsistent with it. First, while pTE proliferation in the blastocyst is regionally limited to those cells in close contact with the ICM, owing to its small size, a similar confinement of growth effected by a local centre of ExE cells in close contact with the epiblast appears at odds with development of the considerably larger ExE. Second, growth of ChE continues steadily despite being separated from the embryo proper by the exocoelomic cavity (Kaufman, 1992).

Interestingly, growth of TSCs depend on the soluble and diffusible FGF4 protein (DelliBovi et al., 1987; Galzie et al., 1997) and a presumably soluble and diffusible feeder-derived factor(s) in vitro (Tanaka et al., 1998), suggesting that TSC progenitors might also be dependent on the fluid-filled proamniotic and, later, the ectoplacental cavity (EPCav) as a source of trophoblast growth promoting substances in vivo. To investigate this hypothesis, the occurrence and persistence of TSC progenitors in trophoblast tissues was examined at, and well beyond, the early-streak stage (approximately 6.5-9.0 dpc).

As in situ identification of TSC progenitors is not possible through either morphological or molecular methods, their whereabouts were addressed in two ways. First, the TSC colony and cell line forming potential of whole dissociated ExE/ChE tissue versus dissociated fractions at the early- and late-streak stages (~6.5-7.5 dpc) was assessed. The persistence of TSC potential was also examined in whole dissociated ChEs at successive stages beyond the late-streak stage (~7.5-9.0 dpc). Second, the number of proliferating cells was identified in histological sections of mitotically arrested ChEs, as TSC progenitors must be included among such mitoses. Our results

indicate that TSC progenitors are not confined to a discrete stratum of ExE in close contact with the epiblast, as had been previously suggested (Tanaka et al., 1998). Instead, a model implicating the EPCav in the sustained growth of TSC progenitors is proposed.

## MATERIALS AND METHODS

### Mice, recovery of conceptuses, in vitro culture and karyotyping

Conceptuses were recovered from PO (Pathology, Oxford) and [C57Bl/6×CBA]F<sub>1</sub> females naturally mated to males of the same genotype, and PO females naturally mated to PO males bred to be homozygous for the ROSA26-β-geo transgene (Friedrich and Soriano, 1991). PO females intended as pseudopregnant recipients for blastocyst transfer to the uterus were mated to vasectomised PO males.

Recovery of blastocysts and post-implantation conceptuses was carried out at room temperature in mouse tubal fluid (MTF)-Hepes medium (Gardner and Sakkas, 1993) or DMEM-based dissecting medium (Lawson et al., 1986), respectively, using established methods (Hogan et al., 1994). Post-implantation conceptuses were staged as detailed elsewhere (Downs and Davies, 1993) or according to the number of somite pairs.

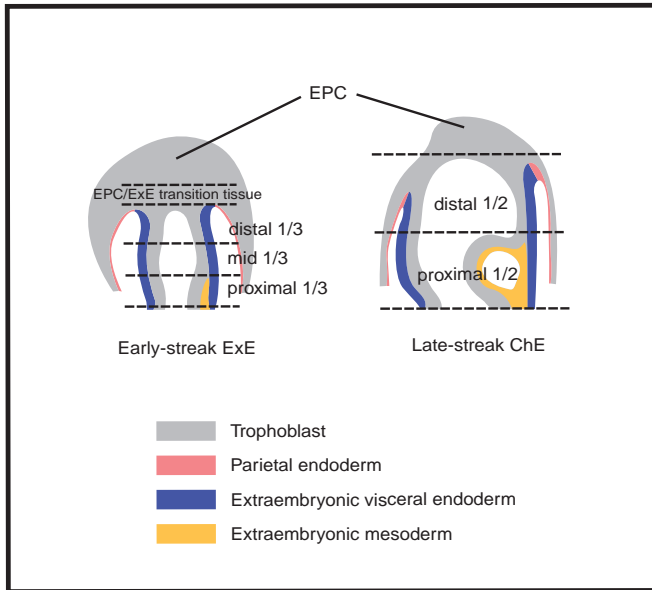
TSC cultures and lines were established from tissues (see below) dissociated by pronase rather than trypsin, as the former produces more complete dissociation (Gwatkin, 1973). Cells were seeded on a confluent feeder monolayer in conditions previously described (Tanaka et al., 1998), with the following modifications. Upon the derivation of some TSC lines, TSC medium was supplemented with 25 ng FGF1/ml (R and D, Minneapolis, MN; 232-FA) instead of FGF4, and cultures were re-fed every 3 days. A TSC culture was classified as a TSC line if it required expansion in tissue culture flasks. No TSC line was classified as such before 2 months of continuous culture. TSC lines were karyotyped as described elsewhere (Hogan et al., 1994). Whole conceptuses and separated extra-embryonic regions (ExRs) (Fig. 1) were isolated (see below) and cultured for 10-20 hours in 1 ml or 0.5 ml roller culture medium, respectively, containing equal parts rat serum and bicarbonate buffered DMEM (Lawson et al., 1986), according to established methods (Cockcroft, 1990).

### Embryonic manipulations, tissue dissociation and histology

#### Preparation of ExE/ChE tissue

Ectoplacental cones (EPCs) were removed and the ExRs isolated and cultured in roller culture or prepared for microdissection into fractions. For the latter series, ExRs were first divested of the extra-embryonic visceral endoderm and any extra-embryonic mesoderm after incubation in a mixture of cold trypsin/pancreatin solution for approximately 10 minutes, as previously outlined for epiblast isolation (Hogan et al., 1994). Although the extra-embryonic visceral endoderm and extra-embryonic mesoderm could be removed cleanly until the late-streak stage, invariably, some of these cells remained attached to a proportion of ChEs beyond this stage, as judged from the presence of cuboidal and rounded cells after their explantation in culture.

Whole ExE/ChE tissues were then dissociated first by incubation at 37°C in pre-equilibrated drops of 0.25% (w/v) pronase (Calbiochem, Nottingham, UK; 537088) in Ca<sup>2+</sup>/Mg<sup>2+</sup>-free Tyrode's saline for approximately 10 minutes and then in Ca<sup>2+</sup>-free ovum culture (OC) medium containing 0.02% (w/v) EGTA for 30 minutes, in a humidified atmosphere of 5% CO<sub>2</sub> in air, as described earlier for the dissociation of epiblast (Gardner and Davies, 2000). Finally, tissues were dissociated in separate TSC culture wells by repeated aspiration through a glass pipette heat-polished to an inner diameter of ~30 μm (early- and late-streak ExE/ChEs) or 100 μm (ChEs beyond



**Fig. 1.** ExE/ChE microdissection. ExRs were microdissected from the conceptus and extra-embryonic visceral endoderm and extra-embryonic mesoderm were removed by trypsin/pancreatin treatment. Proximal and distal are as indicated. ExEs and ChEs were microdissected into fractions perpendicular to the proximodistal axis with a glass needle. Individual ExE/ChE fractions were explanted into separate TSC culture wells following dissociation with pronase, while EPC/ExE transition tissues were explanted as whole, loosely dissociated masses.

the late-streak stage) made as holding pipettes (Gardner and Davies, 2000). Dissociation resulted mainly in single cells, however, two, three and four multi-cell aggregates were also produced.

Following removal of the extra-embryonic visceral endoderm, some early-streak ExEs were microdissected into thirds, and late-streak ChEs into halves, along their proximodistal axis with a glass needle, as illustrated in Fig. 1. Upon incubation in pronase and OC medium, fractionated ExEs were first transferred to pre-equilibrated drops of TSC medium for 10 minutes, then dissociated by repeated aspiration as above. Dissociated cells from individual ExE thirds were then transferred to TSC culture wells and counted as they settled onto the feeder monolayer. Any multi-cell aggregates were recorded as single cells.

Tissue situated immediately out of contact with the proamniotic cavity in early-streak conceptuses was isolated (EPC/ExE transition tissue; Fig. 1) and, after a 5 minute incubation in individual pre-equilibrated drops of 0.25% trypsin/EDTA, was repeatedly aspirated through a glass pipette heat polished to an inner diameter of  $\sim 30 \mu\text{m}$ . Then, tissues were explanted in separate TSC culture wells. This method served only to loosen cell contacts and did not achieve full cell dissociation.

For the Trypan Blue exclusion test, after dissociation of ExE/ChE tissues as above, groups of five same stage tissues were pooled in 50  $\mu\text{l}$  of phosphate-buffered saline (PBS) and then transferred to 15  $\mu\text{l}$  of 0.4% Trypan Blue solution (Sigma, T-8154) for 10 minutes at room temperature. Next, as much of the drop as possible was transferred under the coverslip of a haemocytometer and the mean percentage (number of stained cells/total cells  $\times 100\%$ ) of blue cells from each sample was taken from at least 10 large squares of the haemocytometer coverslip.

#### Preparation of allantoises

Whole 4 somite pair (s.p.) and 8 s.p. allantoises were removed intact as described earlier (Downs and Gardner, 1995). Allantoises

recovered from ROSA26 heterozygous mice were retained. Some of these were then divided into distal (tip) and proximal (base) halves at the 4 s.p. stage using a glass needle (Downs and Harmann, 1997). Whole allantoises or their distal tips were pooled in groups of four and explanted in individual TSC culture wells the day before TSC lines were dissociated and co-cultured with them.

ChE 'median' and 'perimeter' tissue was dissected from individual 6 s.p. ChEs with glass needles. With the ChE lying flat in the bacteriological dish, its median tissue was taken to be its central region, where it had made contact with the EPC. The remaining tissue was considered to be the perimeter of the ChE and was isolated with several cuts. Perimeter segments from individual conceptuses were pooled, dissociated by pronase and plated in TSC culture as usual.

#### Culture of ChEs in the absence of the EPC

EPCs were removed from whole late-streak conceptuses with a glass needle, thereby exposing the EPCav. In those conceptuses in which the EPC was not removed, the EPCav was punctured with a glass needle whose tip was broken by pressing it against the floor of a bacteriological dish containing roller culture medium. After breaking through the ChE, roller culture medium was gently blown into the EPCav until the ChE expanded slightly, when the glass needle was withdrawn. Operated conceptuses were then cultured in roller culture conditions for up to 20 hours (see above). After culture, ChEs were dissociated by pronase as usual and plated in separate TSC culture wells.

#### Production of TSC chimaeras

Chimaeras were produced by blastocyst injection of 15-20 ROSA26 $^{+/-}$ TSCs as described elsewhere (Gardner and Davies, 2000). TSC cultures and conceptuses were photographed in a dissecting microscope with Tungsten corrected film (64T; Kodak).

#### Mitotic index

Colcemid arrest, histology and photography of histological sections were carried out as previously detailed (Downs and Bertler, 2000). The mitotic index of ChE was calculated at the early-bud through late-headfold stages, as these were the only stages when cells of the ChE could be distinguished from those of the EPC.

#### In situ single cell microinjection

Individual ExE cells of  $\sim 7.0$  dpc conceptuses were labelled with  $\sim 4\%$  10 kDa Fluorescein-dextran-lysine (Molecular Probes, Eugene OR) made up in 0.1 M KCl and delivered using the apparatus described elsewhere (Gardner et al., 1992) by inducing a high-frequency oscillation voltage across the electrode resistance (Mobbs et al., 1987). As visualisation of ExE cells through the overlying visceral endoderm was poor, the tissue was first isolated by dissection of isolated ExRs that had been incubated in 0.5% trypsin/2.5% pancreatin in  $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free Tyrode's saline for up to 11 minutes at  $4^\circ\text{C}$ .

#### RNA analysis

Primers for the diploid trophoblast markers *Fgfr2* and *Cdx2*, in addition to  $\beta$ -actin (positive control) were designed to span a large intron of genomic DNA in order to ensure that amplifiable cDNA, but no genomic DNA was present in each tissue sample. Thus, any DNA contamination could be easily detected as the larger band when the PCR products were separated by 1.5% (w/v) agarose gel electrophoresis. The primers and PCR conditions were as follows:

$\beta$ -actin forward, 5' GGC CCA GAG CAA GAG AGG TAT CC 3';  $\beta$ -actin reverse, 5' ACG CAC GAT TTC CCT CTC AGC 3' (annealing temperature  $68^\circ\text{C}$  for 10 cycles followed by  $64^\circ\text{C}$  for 25 cycles; size of product, 460 bp).

*Fgfr2* forward, 5' GAC AAG CCC ACC AAC TGC ACC 3'; *Fgfr2* reverse, 5' CGT CCC CTG AAG AAC AAG AGC 3' (annealing temperature  $66^\circ\text{C}$  for 10 cycles followed by  $63^\circ\text{C}$  for 30 cycles; size of product, 217bp).

**Table 1. TSC colonies and lines isolated from thoroughly dissociated early-streak ExEs and late-streak ChEs, whole or after microdissection into fractions**

Tissue	Number of TSC colonies/total number of TSC colony positive tissues (mean±s.e.m.)	Number of TSC lines derived/number of colonies tested (%)
Early-streak EPC/ExE transition tissues	0/18	–
<b>Early-streak ExEs*†</b>		
Distal 1/3	15/17 (0.9±0.3)	2/8 (25.0)
Mid 1/3	30/17 (1.8±0.5)	2/9 (22.2)
Proximal 1/3	17/17 (1.0±0.4)	1/4 (25.0)
Whole	99/19 (5.2±0.6)	n.d.
<b>Late-streak ChEs*‡</b>		
Distal half	65/27 (2.4±0.2)	4/18 (22.2)
Proximal half	161/27 (6.0±1.1)	6/21 (29.0)
Whole	288/23 (12.5±2.2)	n.d.

s.e.m., standard error of the mean; –, not applicable; n.d., not determined.  
 \*Excluding TSC colony negative fractionated or whole tissues.  
 †Seventeen out of 32 (53.1%) fractionated ExEs were TSC colony positive in at least one of the distal, mid and proximal thirds, while the remaining 15/32 (46.9%) ExEs were TSC colony negative in each third. Four out of 32 (12.5%) fractionated ExEs were TSC colony positive in each third. One out of 20 (0.05%) whole ExEs were TSC colony negative.  
 ‡Twenty-seven out of 31 (87.1%) halved ChEs were TSC colony positive in at least one of the distal or proximal halves, while the remaining four out of 31 (12.9%) ChEs were TSC colony negative in both halves. Thirteen out of 31 (41.2%) halved ChEs were TSC colony positive in each half. None out of 23 whole ChEs were TSC colony negative.

Cdx2 forward, 5' GCA GTC CCT AGG AAG CCA AGT GA 3'; Cdx reverse, 5' CTC TCG GAG AGC CCA AGT GTG 3' (annealing temperature 67°C for 10 cycles followed by 65°C for 30 cycles; size of product, 162 bp).

These unpublished sequences were kindly provided by Tilo Kunath. The specificity of the primer sequences was verified by a BLAST search.

### Statistical analysis

Data were analysed for statistical significance ( $P < 0.05$ ) using a two-way Student's *t*-test, with a 95% confidence interval, assuming equal variances.

## RESULTS

### Derivation of TSC colonies and lines from fractionated post-implantation trophoblast tissues

To investigate whether TSC potential is confined to distinct regions within the developing diploid epithelial ExE/ChE, early-streak ExEs and late-streak ChEs were microdissected into segments along their proximodistal axis (Fig. 1) and seeded in TSC culture. ExEs provided TSC colonies in at least one of each of the distal, mid and proximal thirds from 17/32 (53.1%) ExEs. The mean frequency of TSC colonies derived from TSC positive thirds indicates that each ExE third is capable of producing TSC colonies (Table 1). However, that TSC colonies were absent from each third of the remaining 15/32 (46.9%) ExEs, while only 1/20 (0.05%) dissociated whole ExEs were TSC colony-negative suggests that microdissection into thirds

**Table 2. Mean percentage of TSC colonies derived from dissociated ExE thirds**

Experiment number	ExE number	Number of TSC colonies/number of ExE cells plated		
		Distal third (%)	Mid third (%)	Proximal third (%)*
1	1	3/39 (7.7)	6/76 (7.9)	3/91 (3.3)
	2	1/61 (1.6)	6/129 (4.7)	3/90 (3.3)
2	3	0/40	2/58 (3.4)	1/41 (2.4)
	4	0/88	1/34 (2.9)	0/114
	5	0/63	1/31 (3.2)	0/106
	6	0/98	1/150 (0.7)	1/47 (2.1)
3	7	2/67 (3.0)	0/73	2/47 (4.3)
	8	0/88	2/96 (2.1)	0/101
4	9	0/84	2/92 (2.2)	0/49
	10	2/78 (2.6)	0/67	0/84
	11	0/56	1/96 (1.0)	0/96
5	12	0/68	2/195 (1.0)	0/45
	13	2/118 (1.7)	3/172 (1.7)	6/139 (4.3)
6	14	1/76 (1.3)	1/211 (0.4)	0/105
	15	1/156 (0.7)	0/147	0/281
	16	3/156 (1.9)	1/94 (1.0)	1/256 (0.4)
7	17	0/65	1/52 (1.9)	0/156
Mean percentage of TSC colonies±s.e.m.†		1.2±0.5%	2.0±0.5%	1.2±0.4%
Number of TSC colony positive thirds/ExEs dissociated (%)‡		8/17 (47.1)	14/17 (82.4)	7/17 (41.2)

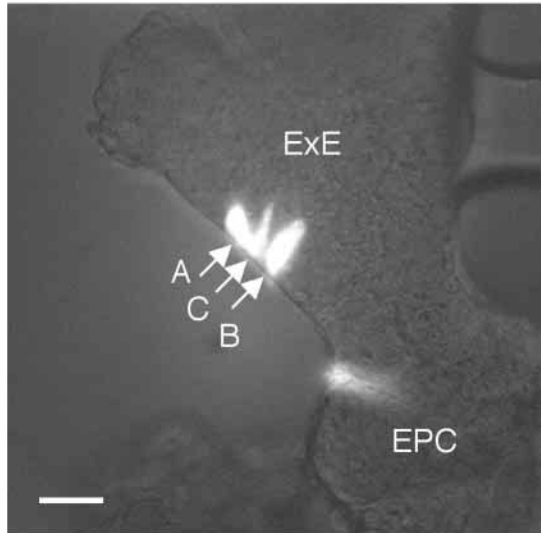
s.e.m., standard error of the mean.  
 \*Proximal third cell count included a small number of epiblast and extra-embryonic mesoderm cells.  
 †The mean percentages for proximal versus mid, mid versus distal and distal versus proximal thirds were not significantly different at  $P > 0.05$ .  
 ‡The percentage of TSC colony positive mid thirds was significantly higher than either the proximal or distal thirds at  $P < 0.05$ . However, the percentage of TSC colony positive proximal versus distal thirds was not significantly different at  $P > 0.05$ .

impairs TSC colony forming potential in the ExE. Only four out of 32 (12.5%) microdissected ExEs exhibited TSC colonies in all thirds (Table 1).

Similarly, ChEs provided TSC colonies in at least one of each of the proximal and distal halves in 27/31 (87.1%) cases (Table 1). Furthermore, the mean frequency of TSC colonies obtained from TSC positive halves indicates, as with the ExE, that TSC colonies can be produced from a wide domain of diploid trophoblast. However, in contrast to the ExE, in only four out of 31 (12.9%) ChEs were both halves TSC colony negative. Moreover, in 13/31 (41.2%) bisected ChEs, both halves were TSC colony positive (Table 1).

To confirm the long-lived mitotic potential of TSC colonies obtained from each ExE/ChE fraction, those from each ExE/ChE segment were repeatedly passaged to form TSC lines. Similar frequencies of TSC lines were obtained from each ExE third and ChE half (Table 1). In contrast to the TSC colony-positive ExE and ChE, none of the 18 EPC/ExE 'transition' tissues – an area presumably rich in diploid trophoblast, yet which does not exhibit an epithelial morphology (Fig. 1) – retained an undifferentiated morphology during culture and when passaged, none produced TSC colonies (Table 1).

To determine if each region of the ExE contributed similar numbers of TSC colonies, dissociated ExE cells seeded for the



**Fig. 2.** Non-coherent growth of ExE. Isolated day 7 ExE several minutes after cell A was injected with fluorescein-dextran-lysine. Note that cell B, though clearly not adjacent to A, has become about as strongly labelled as A and must therefore be its sister. The more weakly labelled intervening cell, C, is presumably a product of the division immediately preceding the one yielding A and B. Scale bar: 50  $\mu$ m.

data in Table 1 were counted before plating. The mean frequency of TSC colonies obtained from dissociated TSC colony positive thirds was calculated as a percentage of the number of cells plated. Although the percentage of TSC colonies did not vary significantly between regions within experiments, nonetheless, when data between experiments were compared, a significantly higher proportion of mid compared with distal or proximal thirds was TSC colony positive (Table 2). This might be explained by the possibility that, upon microdissection of the ExE/ChE, more cells happened to be mitotically active at the time of isolation in the mid-portion than in either of the other two thirds. Cell counts were not made on dissociated ChE tissue because the numbers were too large to allow this to be carried out with acceptable accuracy.

### Non-coherent growth of ExE cells

Growth of the early-streak ExE was analysed by *in situ* labelling of single cells to investigate the possibility that cells with TSC colony and cell line forming potential become widely dispersed because the ExE grows non-coherently at the onset of gastrulation, as does epiblast tissue (Gardner and Cockroft, 1998). Single ExE cells were microinjected with 10 kDa fluorescein-dextran-lysine in order to determine the spatial relationship between products of recent mitoses from the pattern of spread of the label (Fig. 2).

As shown in Table 3, the overall incidence of spread of label for ExE of 19% was about half that found earlier in seventh dpc epiblast (38%) (Gardner and Cockroft, 1998), but the proportion of cases where spread was to a non-adjacent cell was higher (seven out of 12 as opposed to 18/37 for epiblast). This suggests that ExE cells grow non-coherently and supports the view that TSC progenitors become extensively distributed throughout the ExE/ChE as a result of random cell mixing. The overall impression was that the arrangement of cells was less

**Table 3.** *In situ* labelling of single ExE cells in day 7 conceptuses

Total ExEs	Total cell fills	Total number with spread to one or more cells	Number with spread to immediately adjacent cell	Number with spread to non-adjacent cell*
14	64	12	5	7

\*Included in this category were four cases where directly and indirectly labelled cells were played well apart, being separated by intervening unlabelled cells over almost their entire length, and, thus, retained only focal contact.

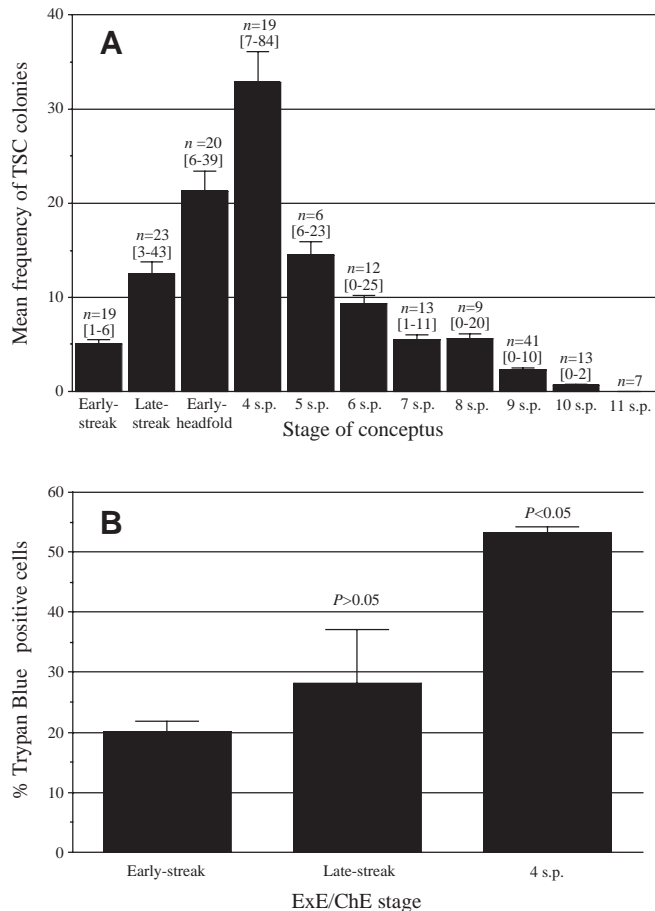
ordered than in the epiblast, consistent with their moving or being displaced more rapidly during tissue growth. If true, this would be expected to reduce the time for which the intercellular bridges formed during cytokinesis remain patent, and could thus explain why the incidence of spread of label was lower than recorded for the epiblast.

### Persistence of cells with TSC potential during post-implantation development

The close apposition of the proximal and distal aspects of the ChE beyond the late-streak stage prevented its bisection perpendicular to the proximodistal axis, as was possible for the ExE. Instead, ChEs beyond the late-streak stage were dissociated as whole tissues to determine the approximate stage of development when TSC derivation is no longer possible. The mean frequency of TSC colonies derived from conceptuses steadily increased until the four somite pair (s.p.) stage, after which it began to decline (Fig. 3A). TSC colonies were absent from 8.3% 6 s.p. ChEs ( $n=12$ ) and from 11.1% 8 s.p. ChEs ( $n=9$ ). At later stages, this frequency increased to 65.9% 9 s.p. ChEs ( $n=41$ ), 61.5% 10 s.p. ChEs ( $n=13$ ) and 100% 11 s.p. ChEs ( $n=7$ ).

To investigate whether the initially high frequency of TSC colony formation from ExE/ChE might be due to preferential survival of their progenitors during dissociation, instantaneous cell death was estimated. Dissociated cells from whole early-streak ExEs, late-streak ChEs and 4 s.p. ChEs were exposed to Trypan Blue, which is taken up selectively by dead cells. Because the highest percentage of dead cells (Fig. 3B) overlapped with the time when the highest numbers of TSC colonies were formed (Fig. 3A), at the 4 s.p. stage, we concluded that TSC forming potential is not due to preferential survival of TSC progenitors. Thus, these findings imply that the number of cells with TSC potential expands during the initial stages of ChE formation.

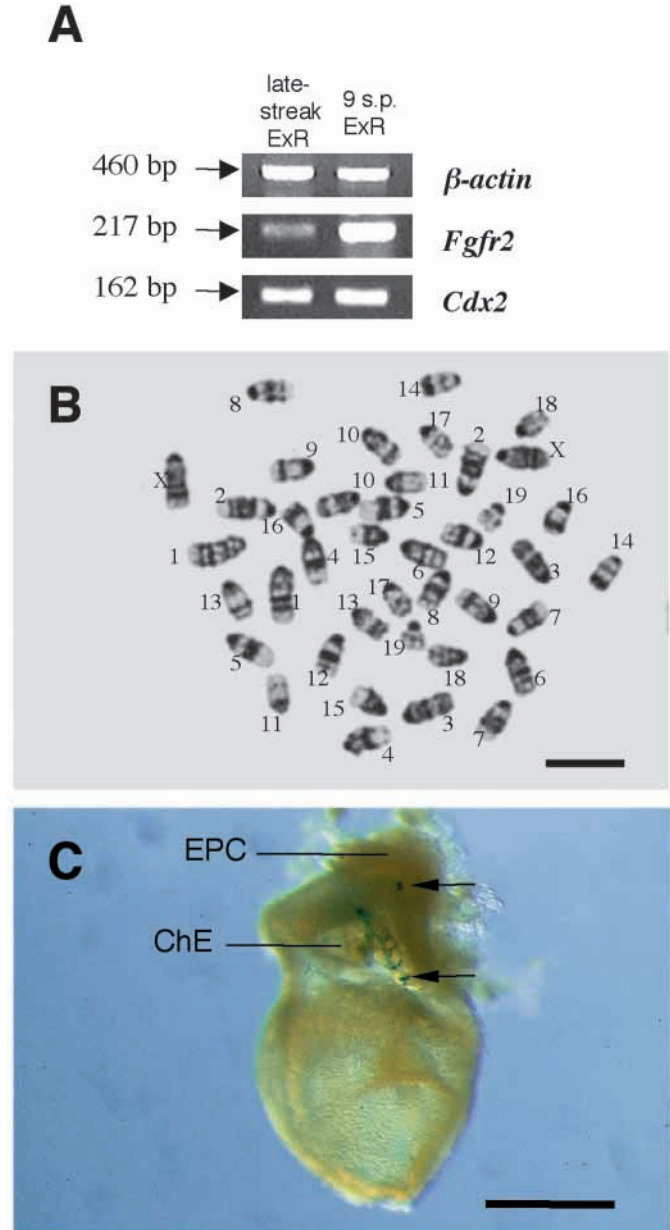
The failure of previous *in situ* hybridisation analyses to detect expression of diploid trophoblast markers: *Eomes* (Ciruna and Rossant, 1999), *Cdx2* (Beck et al., 1995) and *Err2* (Pettersson et al., 1996) in the ChE beyond the early-headfold stage might suggest that TSC potential exhibited by ChE cells *in vitro* beyond this stage (Fig. 3A) occurs in cells that do not normally possess this potential *in vivo*. Hence, expression of *Cdx2*, in addition to that of *Fgfr2*, a gene apparently necessary for the maintenance of TSC progenitors (Kunath et al., 2001), was assessed by sensitive RT-PCR in the 9 s.p. extra-embryonic region (ExR). Given the sensitivity of RT-PCR to detect transcripts from as little as a single cell (Dixon et al., 2000), this region was classified as the 'ExR', as it was not possible to be sure that isolated ChE was devoid of contaminating extra-



**Fig. 3.** TSC colonies and cell death from dissociated whole ExE and ChE throughout post-implantation development. (A) TSC colonies derived from dissociated whole ExE/ChEs throughout stages in post-implantation development. The mean frequency of TSC colonies was determined as the number of TSC colonies/number of tissues dissociated. The range of TSC colonies obtained from individual tissues is indicated in square brackets. (B) The mean percentage of instantaneous cell death in ExE/ChEs after dissociation, as determined by the Trypan Blue exclusion test. The percentage of Trypan Blue-positive cells was calculated as a function of total cells. A significant increase in cell death was observed between late-streak ChEs and 4 s.p. ChEs at  $P < 0.05$ . However, there was no significant difference between early-streak ExEs and late-streak ChEs at  $P > 0.05$ . Error bars indicate s.e.m.

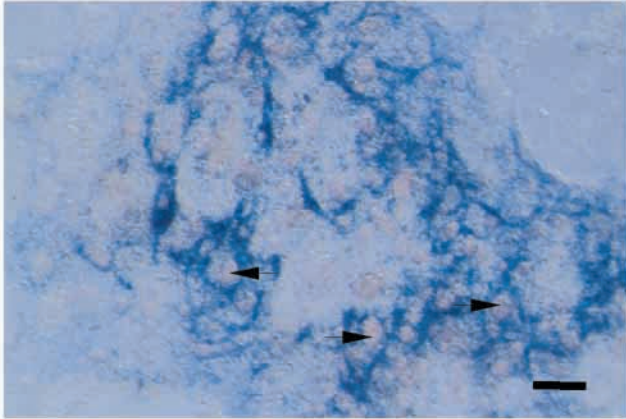
embryonic visceral endoderm or mesoderm cells. The ExE/EPC transition region was not assayed as a negative control as it is presumably rich in diploid cells, as these cells are present in the EPC core and ExE (Ilgren, 1981). Transcripts were detected from both late-streak (positive control) and 9 s.p. ExRs for each gene analysed (Fig. 4A). As these markers are purported to be diploid trophoblast specific in the ExR on the basis of mutational and expression analyses (Kunath et al., 2001), it was assumed that the ChE was the source of the transcripts, rather than potentially contaminating extra-embryonic visceral endoderm and/or mesoderm.

To confirm that cells with TSC potential can exist as late as the 9 s.p. stage in some conceptuses, a chimaeric analysis was undertaken. Two TSC positive cultures, obtained from separate 9 s.p. ChEs heterozygous for the ROSA26- $\beta$ -geo transgene,



**Fig. 4.** Analysis of TSC potential in the 9 s.p. ChE. (A) RT-PCR analysis of TSC markers in late-streak and 9 s.p. ExRs.  $\beta$ -actin is a positive control. The sizes of the PCR products are indicated on the left. (B) Photomicrograph of a 40 XX chromosome spread from a representative cell of the polyclonal TSC line, derived from a single 9 s.p. ChE after approximately 2 months in culture, used for chimaeric analysis. Numbers represent specific chromosomes. Scale bar: 10  $\mu$ m. (C) Photomicrograph of a late-headfold stage TSC chimaera produced by blastocyst injection of the 9 s.p. TSC line in B. Arrows indicate contribution of blue staining ROSA26 +/- TSCs to the ChE and EPC. Scale bar: 500  $\mu$ m.

were expanded as polyclonal TSC lines for blastocyst injection. The karyotype of one of these lines was abnormal and was therefore omitted from further characterisation (data not shown). The karyotype of the second line was predominantly euploid and XX (Fig. 4B). However, ~20% of the chromosome spreads were tetraploid, consistent with the spontaneous



**Fig. 5.** Photomicrograph of 4 s.p. allantoic distal tips in co-culture with TSC colonies. After 9 days in TSC culture, TSC colonies (arrows) from a line derived from a wild-type early-streak ExE continued to proliferate on, and in close proximity to, blue ROSA26 +/- allantoic tissue. Scale bar: 200  $\mu$ m.

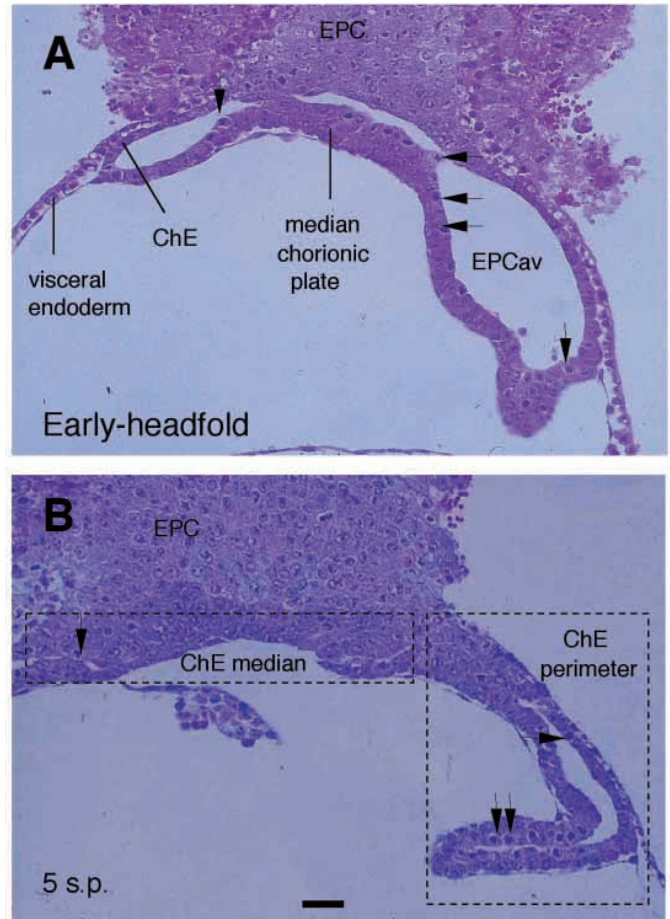
differentiation of TGCs in TSC lines (Tanaka et al., 1998). Furthermore, in two exceptional cells, a reciprocal translocation was observed between chromosomes 4 and 8, in addition to a deleted chromosome 6 (data not shown). Nevertheless, as judged by the ability of most of the TSC colonies to stain blue in the presence of X-gal, this line expressed the ROSA26- $\beta$ -geo transgene (data not shown). It was, therefore, injected at early passages, into blastocysts with the aim of producing chimaeras.

Of 27 blastocysts transferred to the uterus, 24 (88.9%) were recovered between late-streak and early-headfold stages of which four (16.7%) were chimaeric. In the chimaeras, donor cells contributed exclusively to trophoblast tissues, including the ChE, EPC and secondary TGCs of the parietal yolk sac. However, the number of blue patches varied from in excess of a dozen (Fig. 4C) to as few as two (data not shown).

### The role of the allantois in the persistence of cells with TSC potential

The mean frequency of TSC colonies began to decline between the 4 and 5 s.p. stages which is when the allantois begins to fuse with the chorion (Downs and Gardner, 1995). This observation raised the possibility that the allantois might inhibit TSC potential in the ChE. To test the role of the allantois in inhibiting TSC potential, three series of experiments were carried out.

In series 1, allantoises that had not yet made physical contact with the ChE were removed from conceptuses by aspiration at the 4 s.p. stage. These otherwise intact conceptuses were then roller cultured for 10 hours (Table 4). Wherever possible, intact late-streak conceptuses were cultured in parallel as positive controls. Two exceptional 4 s.p. experimental ChEs in this series failed to develop beyond the 4 s.p. stage, while the remaining 25 ChEs developed to a final stage of between 5-7 s.p.. After culture, ChEs were dissociated as usual and plated to examine TSC colony forming potential. Intact ChEs initially at the late-streak stage provided a significantly higher mean frequency of TSC colonies than ChEs initially at 4 s.p. (Table 4), suggesting that factors other than allantoic contact with the chorion leads to diminution in TSC forming potential over time.



**Fig. 6.** Distribution of mitotic cells in the developing ChE. (A,B) Representative photomicrographs of sagittally sectioned, mitotically arrested ChEs. Examples of mitotic cells are indicated by arrows. Note that mitotic cells were located throughout the ChE, but lay predominantly apically in the chorionic plate in the early-headfold ChE. (A) Early-headfold ChE that has not yet undergone fusion. (B) 5 s.p. ChE that has undergone fusion. Arrow in the ChE median box indicates an exceptional mitotic cell. Scale bar: 25  $\mu$ m.

In series 2, 4 s.p. ExRs, the ChEs of which had not yet made physical contact with the allantois, were removed from conceptuses and cultured in roller culture for 10 or 20 hours to promote their advanced development. Isolated late-streak ExRs were cultured in parallel as positive controls when possible. The mean frequency of TSC colonies derived from ChEs initially at the 4 s.p. stage post-culture was significantly lower than those derived from late-streak controls for both time intervals (Table 4), again suggesting that contact with the allantois is not responsible for declining TSC potential.

In series 3, whole 4 s.p. and 8 s.p. allantoises as well as 4 s.p. allantois 'distal tips' were isolated from conceptuses heterozygous for the ROSA26- $\beta$ -geo transgene and explanted under TSC conditions on a feeder monolayer on which they began to spread after 1 day. Cells of a TSC line derived from a wild-type ExE at the early-streak stage were then seeded directly on this flattened allantoic tissue (Fig. 5). Altogether, four cultures were established from whole 4 s.p. allantoises, six cultures from whole 8 s.p. allantoises and four cultures

**Table 4. Effect of 10 or 20 hour roller culture on the mean frequencies of TSC colonies isolated from late-streak and 4 s.p. ChEs, in the absence of the allantois**

Final stage	Mean frequency of TSC colonies (number of TSC colonies/number of tissues analysed $\pm$ s.e.m.)			
	Initial stage			
	Series 1*		Series 2*	
	Whole conceptuses, 10 hour culture		Isolated ChEs, 10/20 hour culture	
	4 s.p., extracted allantoises	Late-streak (controls)	4 s.p.	Late-streak (controls)
N.D.	–	–	3.9 $\pm$ 1.4 [0-19] 10 hours <i>n</i> =17	10.1 $\pm$ 1.9 [6-20] 10 hours <i>n</i> =12
			1.7 $\pm$ 0.5 [0-8] 20 hours <i>n</i> =21	14.5 $\pm$ 3.5 <sup>†</sup> [10-36] 20 hours <i>n</i> =8
Early- to late-bud	–	39.0 $\pm$ 6.7 [28–45] <i>n</i> =3	–	–
Early-headfold	–	17.7 $\pm$ 2.7 [6–25] <i>n</i> =7	–	–
4 s.p.	3.5 $\pm$ 3.5 [1-6] <i>n</i> =2	–	–	–
5 s.p.	3.3 $\pm$ 1.2 [0–8] <i>n</i> =7	–	–	–
6 s.p.	1.4 $\pm$ 0.5 [0–5] <i>n</i> =12	–	–	–
7 s.p.	0.5 $\pm$ 0.2) [0–1] <i>n</i> =6	–	–	–

Range of TSC colonies obtained from individual tissues are indicated in square brackets.

s.e.m. standard error of the mean; s.p., somite pair; *n*, number of tissues analysed; –, not applicable; N.D., not determined.

\*There was a significant increase in the mean frequency of TSC colonies derived post-culture from late-streak ChEs ab initio versus 4 s.p. ChEs ab initio, at  $P < 0.001$ , in series 1 and 2.

<sup>†</sup>One technical failure omitted.

from 4 s.p. allantois 'tips'. As a result of co-culture, TSC proliferation did not appear to be impaired as for the duration of the culture period, undifferentiated flattened TSC colonies continued to grow in the presence of allantois explants (Fig. 5). Together, the results in these three series suggest that TSC potential is lost independently of the presence of the allantois.

### Distribution of mitotic cells and cells with TSC potential in the ChE

To define the whereabouts of TSC progenitors in the ChE more precisely, TSC potential should ideally be examined in single ChE cells in vivo. Given that cell division is a property associated with multi-potency (Kunath et al., 2001), we reasoned that the population of mitotic cells should include TSC progenitors. Conceptuses at, and beyond, the early-bud stage were treated with the mitotic inhibitor colcemid to enrich for mitotic ChE cells (Downs and Bertler, 2000). Treated and control conceptuses recovered between the neural plate and headfold stages were prepared for histology and examined by brightfield microscopy.

It was found that in early-allantoic bud specimens, where the chorion had not made any contact with the EPC, the mitotic

index was as high throughout the ChE (Fig. 6A), as it was in the contact-free regions of more advanced specimens (Fig. 6B and 19.5 $\pm$ 2.1; Table 5). By contrast, once the median ChE had contacted the EPC so that the EPCav was occluded locally, its mitotic index was significantly lower (7.4 $\pm$ 1.1; Table 5) than in the peripheral regions that had not made contact with the EPC (17.2 $\pm$ 1.6; Table 5).

The relative decline in mitoses in the ChE median versus perimeter, suggests that there may also be a difference in TSC potential between these two regions. To this end, ChE median and perimeter tissue were isolated from individual 6 s.p. ChEs – a stage at which 100% of ChEs were observed to have undergone fusion in the median but not in the perimeter (Downs, 2002) – and plated in separate TSC culture wells. The mean frequency of TSC colonies derived from the unfused ChE perimeter was significantly higher than that from the fused ChE median (see Fig. 7).

### Perturbation of EPCav occlusion and the persistence of TSC potential

These findings suggest that occlusion of the EPCav by union between ChE and EPC may account for the observed decline



**Table 5. Mitotic index of the ChE**

Stage	With (+) or without (-) colcemid	Status of chorionic ectoderm	Number of specimens scored	Mitotic index $\pm$ s.e.m.	<i>P</i> -value (Student's 2-way <i>t</i> -test)
Early-bud	+	Unfused	3	18.9 $\pm$ 2.9	N/A
	-	Unfused	2	3.6 $\pm$ 0.8	N/A
Late-bud	+	Unfused	3	15.6 $\pm$ 2.4	0.034
	+	Fused	3	5.8 $\pm$ 2.0	
	-	Unfused only	1	3.2	N/A
	-	Unfused	1	2.0	N/A
Early-headfold	-	Fused	1	1.0	
	+	Unfused	3	19.0 $\pm$ 2.8	0.02
	+	Fused	3	7.8 $\pm$ 1.2	
	-	Unfused	3	7.1 $\pm$ 4.4	0.44
-	Fused	3	3.1 $\pm$ 1.4		
Late-headfold	+	Unfused only	1	21.2	N/A
	+	Unfused	1	16.6	N/A
	+	Fused	1	10.7	
	-	Unfused	2	3.8 $\pm$ 1.2	0.47
	-	Fused	2	2.6 $\pm$ 0.4	
	Combined	+	Unfused only	4	19.5 $\pm$ 2.1
+		Unfused	7	17.2 $\pm$ 1.6	0.00
+		Fused	7	7.4 $\pm$ 1.1	

s.e.m., standard error of the mean; N/A, not applicable.

The mitotic index was calculated in the ChE of previously prepared specimens as described earlier (Downs and Bertler, 2000). In this study, the ChE was either fused with the EPC ('fused') or not fused ('unfused') (see Fig. 8). Only those specimens where ChE could be unambiguously distinguished from the EPC either by intensity of Haematoxylin stain or by gaps between the two tissues were scored. Mitotic nuclei were scored as those densely staining metaphase chromosomes not contained within a nuclear envelope.

\**P* value for combined chorions that were completely unfused along their length versus peripheral combined unfused chorions whose median region was fused with the EPC.

in TSC potential. To test this possibility, we interfered with the normal disappearance of this cavity by microsurgically removing the EPC from conceptuses at the late-headfold stage, thereby preventing normal ChE fusion to the EPC (series 1). After 10 hours of culture, ChEs were dissociated in order to derive TSC colonies. To control for the possibility that leakage of EPCav fluid out of, and roller culture medium into, the EPCav may inadvertently affect the outcome of this experiment, ChEs of late-streak conceptuses were punctured (series 2). The mean frequency of TSC colonies obtained from ChEs cultured in the absence of the EPC was significantly higher than in either unoperated control conceptuses or conceptuses in which the ChE had been punctured (Table 6).

## DISCUSSION

Our results indicate that TSC progenitors are distributed widely in the ExE, and then the ChE, prior to fusion with the EPC, on the basis of the non-local distribution of TSC colonies and lines in vitro (Table 1 and Table 2), and mitoses in vivo (Fig. 6A and Table 5). These findings are supported by the pattern of ExE cell division that revealed that labelled sister cells were frequently not immediately adjacent to each other (Fig. 2 and Table 3), indicating that considerable cell mixing and dispersal occurs during growth of the ExE/ChE.

**Table 6. Effect of 10 hour roller culture on the mean frequencies of TSC colonies isolated from late-streak ChEs in situ, in the absence of the EPC**

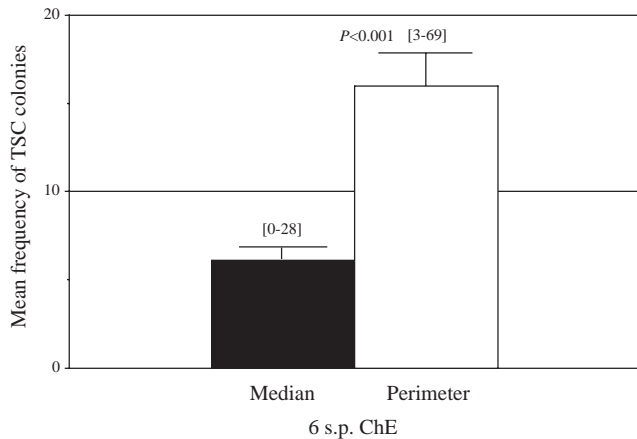
Final conceptus stage	Late-streak conceptuses ab initio, 10 hour culture			
	Series 1*		Series 2†	
	Without EPC	Intact (controls)	Punctured EPCav	Intact (controls)
Early- to late-bud	48.0 $\pm$ 3.2 [10-80] <i>n</i> =4	22.7 $\pm$ 9.4‡ [10-35] <i>n</i> =6	19.8 $\pm$ 8.9 [2-20] <i>n</i> =3	-
Early- headfold	62.6 $\pm$ 5.7‡ [11-93] <i>n</i> =20	32.5 $\pm$ 8.3‡ [3-62] <i>n</i> =4	35.7 $\pm$ 7.6 [10-118] <i>n</i> =19	31.7 $\pm$ 5.3 [16-51] <i>n</i> =8
Late-headfold	54.0 $\pm$ 26.9 [35-73] <i>n</i> =2	-	-	-

Frequency calculated as number of TSC colonies/number of tissues analysed $\pm$ s.e.m.  
Range of TSC colonies obtained from individual tissues is indicated in square brackets.  
*n*, number of tissues analysed; -, data not available.  
\*Mean frequencies of experimental and control conceptuses were significantly different at *P*<0.05.  
†Mean frequencies of experimental and control conceptuses were not significantly different at *P*>0.05.  
‡One technical failure omitted.

The apparent non-coherence of ExE growth invites the suggestion that TSC progenitors originate in the proximal third adjacent to the epiblast, and become displaced distally upon division. However, this is unlikely as the ExE third abutting the epiblast, a region intensely expressing the diploid markers: *Cdx2* (Beck et al., 1995), *Err2* (Pettersson et al., 1996) and *Eomes* (Ciruna and Rossant, 1999) did not produce a significantly higher percentage of TSC colonies (Table 2) or lines (Table 1) than either of the other two thirds under identical conditions, as might be expected if maintenance of TSC progenitors depend on such contact. Moreover, TSC lines could be isolated from the distal ChE at the late-streak stage, ~1 day after the ExE loses contact with the epiblast (Table 1).

Reliance on in situ hybridisation patterns of diploid trophoblast markers as a means of demarcating the TSC progenitor domain in the ExE is questionable as expression of these genes is downregulated beyond the late-streak stage (Beck et al., 1995; Pettersson et al., 1996; Tanaka et al., 1998; Ciruna and Rossant, 1999) when TSC colonies were observed to increase in number in the present study (Fig. 3A,B). Recently, it was reported that *Nodal* signalling in the epiblast, which is believed to occur in response to induction from markers in the abutting proximal ExE, maintains expression of the diploid trophoblast markers *Bmp4* and *Eomes* in this region via a feedback mechanism (Brennan et al., 2001). Moreover, conceptuses bearing a null mutation in *Bmp4*, which is intensely expressed in ExE cells adjacent to the epiblast in undisturbed conceptuses, fail to form primordial germ cells (Lawson et al., 1999). Thus, available evidence suggests these ExE markers are involved in embryonic differentiation rather than in the maintenance of TSC progenitors in the region of ExE adjacent to the epiblast.

That EPC/ExE transition tissue never yielded undifferentiated colonies following dissociation (Table 1)



**Fig. 7.** Distribution of TSC potential in the 6 s.p. ChE. TSC colonies derived from the ChE median and ChE perimeter of 6 s.p. ChEs ( $n=20$ ). Range of TSC colonies obtained from each region of individual tissues are indicated in square brackets. There was a significant difference between the ChE median and perimeter at  $P<0.001$ . One exceptional ChE produced TSC colonies greater in number than three times the mean in each ChE region: ChE median, 28 TSC colonies; ChE perimeter, 69 TSC colonies.

implies that TSC potential is absent distal to the arbitrary boundary of EPC tissue that coincides with the point of insertion of Reichert's membrane (Snell and Stevens, 1966). This is consistent with the irreversible uptake of large latex particles in this region of the EPC, which is claimed to be a property of differentiated trophoblast (Rassoulzadegan et al., 2000). Collectively, available evidence suggests that the TSC 'niche' probably spans the entire ExE/ChE. This is perhaps not surprising as *Fgfr2*, the candidate receptor for FGF4 signalling in diploid trophoblast, is uniformly expressed throughout the ExE/ChE, until at least the 8.0 dpc stage (Orr-Urtreger et al., 1991) and, along with *Cdx2*, *Fgfr2* was detected in the ChE until at least the 9 s.p. stage by non-quantitative RT-PCR (Fig. 4A). Indeed, FGFR2-mediated signalling is necessary for the genesis and maintenance of columnar epithelia in embryoid bodies (Li et al., 2001) and thus may be involved in the longevity of other embryonic epithelia such as the ExE/ChE.

Interestingly, two changes in ChE morphology seem to relate to the relative decline of TSC potential in the ChE median versus perimeter. The first was deduced from a previous study (Downs, 2002): the median ChE while initially flattened, adopts a 'folded' morphology upon EPCav occlusion in this region, whereas a flattened morphology persists in the peripheral ChE regions, where the EPCav is not yet occluded. This is consistent with the observation that the EPC/ExE transition region, a population of diploid trophoblast that does not exhibit an epithelial morphology, failed to provide undifferentiated TSC colonies (Table 1). Moreover, although dissociated pre-trophectoderm cells of the third day compacted morula can yield TSC lines, in order to do so, they must first differentiate into vesiculated trophoctoderm (G. D. U., unpublished). Cumulatively, these observations indicate that the epithelial morphology promotes or facilitates TSC potential. The second observation of note is the disappearance of the EPCav itself (Fig. 6).

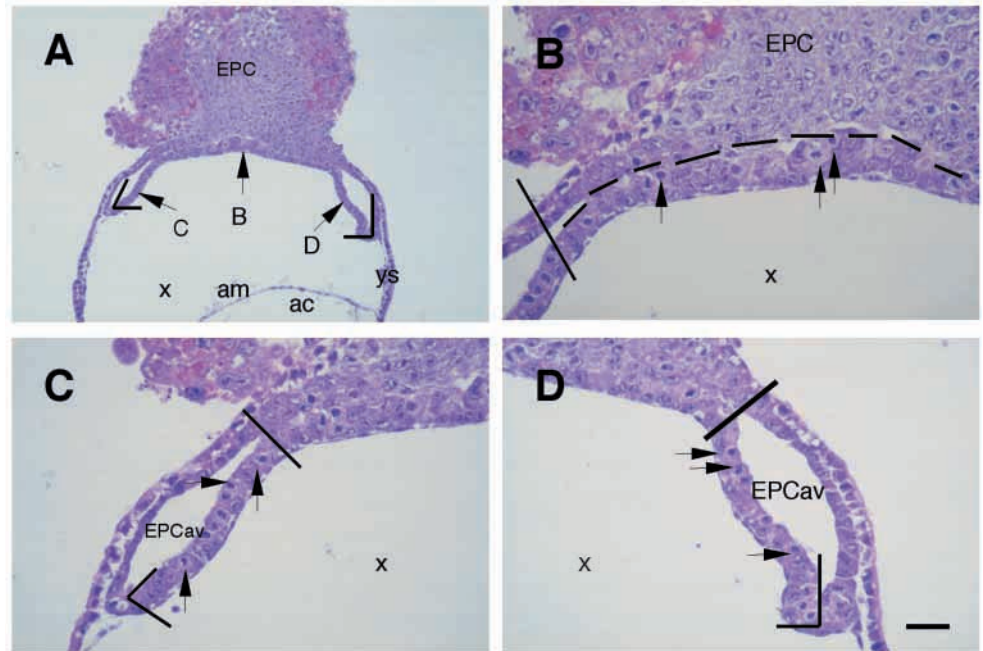
The striking temporal and spatial correspondence between decline of TSC potential (Fig. 7) and ChE mitoses (Table 5)

with occlusion of the EPCav – rather than ChE union with the allantois (Fig. 5 and Table 4) – raises the intriguing possibility that TSC progenitors are negatively regulated by the disappearance of the EPCav rather than by the presence of the allantois, a tissue that has been previously reported to promote differentiation in the ChE (Hernandez-Verdun and Legrand, 1975; Voss et al., 2000). It is worth noting in this context that the ExE shares a common cavity with the epiblast, the proamniotic cavity, during its rapid growth. Even with the formation of the amnion when this is no longer the case, the ChE lines part of the former proamniotic cavity and thus, the EPCav remains a relic of the former proamniotic cavity.

Although it is tempting to assign TSC potential of the ChE to mitotic cells directly facing the EPCav (Figs 6, 8), caution is warranted because the tissue appears to be pseudostratified (Tamarin and Boyde, 1976) and like its precursor ExE (Fig. 2 and Table 3), is likely to grow non-coherently. Hence, the apical location of mitotic cells may be attributable to loss of contact with the basal lamina, as has been demonstrated for other transient embryonic epithelia such as epiblast (Gardner and Cockroft, 1998). Nevertheless, other evidence is consistent with dependence of ChE proliferation on EPCav fluid.

Clearly, diploid trophoblast proliferation is ascribed to a positive signal external to diploid trophoblast as pTE (Gardner and Johnson, 1972) and ExE/ChE (Rossant and Ofer, 1977; Rossant and Tamura-Lis, 1981) tissue in isolation rapidly undergo postmitotic TGC formation, the default state of differentiation of initially diploid trophoblast. Furthermore, that TSC potential in vitro disappeared simply as a consequence of the absence of either FGF4, heparin or feeder conditioned medium (Tanaka et al., 1998) appears to include the possibility that TSC potential may disappear in response to a loss of access to these factors in vivo rather than the provision of a specific induction by the EPC. Thus, undifferentiated TSC progenitors persist while in the presence of a mitotic signal. This conclusion is supported by two findings in the present study. First, interfering with the normal course of EPCav occlusion, by EPC removal at the late-streak stage, promoted a significant numerical increase of TSC colonies as compared to controls (Table 6). Second, the frequency of TSC progenitors steadily increased during undisturbed development until at least the 4 s.p. stage (Fig. 3), which spans a period before EPCav occlusion is observed to occur throughout the entirety of the ChE (Downs, 2002). Together, these observations suggest that TSC progenitors continue to multiply in the ChE unless EPCav occlusion is permitted to occur. By implication, therefore, EPCav fluid is a promising source of growth stimulation for TSC progenitors in undisturbed development.

Expansion of the proamniotic and ectoplacental cavities is thought to be driven by the high osmotic pressure of the fluids they contain. One molecule believed to play a role in generating the necessary osmotic pressure is the glycosaminoglycan, hyaluronan (HA), which is evidently secreted from the epiblast into the proamniotic and ectoplacental cavities (Brown and Papaioannou, 1993). It is therefore conceivable that other molecules, including components of the FGF4 signalling system could also be made available to the ExE/ChE by this means. FGF4 is not only secreted, soluble and diffusible (DelliBovi et al., 1987; Galzie et al., 1997) but its transcripts are very abundant in the epiblast until the onset of gastrulation (Niswander and Martin, 1992).



**Fig. 8.** Scoring mitotic figures in unfused and fused chorionic ectoplacental junctions for data in Table 5. Brightfield photomicrographs of a late-headfold stage exposed to colcemid for 2 hours and prepared for histochemistry (see Materials and Methods). Arrows in B-D indicate examples of mitotic figures in metaphase. (A) The chorionic area scored for mitotic figures is indicated between the 'brackets'. The three arrows point to the region of ChE shown in B-D. (B) ChE median of A showing the fused chorionic area beneath the broken lines and bordered on the left by a unbroken black line. (C) Unfused ChE perimeter to the left of the unbroken black line of B, the extent of which is enclosed by a 'bracket'. (D) Unfused ChE perimeter to the right of the fused ChE median, the extent of which is also enclosed by a 'bracket'. Abbreviations: ac, amniotic cavity; am, amnion; EPCav, ectoplacental cavity; EPC, ectoplacental cone; x, exocoelomic cavity; ys, yolk sac. Scale bar: 100  $\mu$ m for A; 25  $\mu$ m for B-D.

However, although spatiotemporal expression patterns are available for *Fgf4* (Niswander and Martin, 1992; Rappolee et al., 1994; Brison and Schultz, 1996) and its candidate receptor, *Fgfr2* (Orr-Urtreger et al., 1991; Orr-Urtreger et al., 1993), there is relatively little information on the distribution of proteoglycans that could enable FGF4 to function as a ligand. Some insight pertaining to the latter issue is available in other systems. Proteoglycans have been detected in human follicular fluid (Eriksen et al., 1999), and heparan sulfate in particular, which is secreted by granulosa cells in vitro (Yanagishita and Hascall, 1979), has been shown to occur in both human (Eriksen et al., 1997) and bovine follicular fluid (Bellin and Ax, 1987).

Finally, it is important to consider that EPCav fluid and blastocoelic fluid of the blastocyst may be distinct. Clearly, the fate of the mural trophoblast, in contact with blastocoelic fluid, is to differentiate into TGCs. This discrepancy might be explained by the fact that blastocoelic fluid does not emanate from embryonic tissues. Rather, it consists mainly of water, whose purpose is to achieve cavitation of the morula (Watson, 1992). Collectively, the results of this study challenge the notion that physical contact with embryonic tissue is essential for the proliferation of undifferentiated diploid trophoblast upon the formation of the egg cylinder. Although involvement of extra-embryonic endoderm or mesoderm cannot be discounted at this juncture, the possibility that trophoblast growth is controlled via factors contained within EPCav clearly warrants further investigation.

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