Imprinted X-inactivation in extra-embryonic endoderm cell lines from mouse blastocysts

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Summary

The extra-embryonic endoderm lineage plays a major role in the nutritive support of the embryo and is required for several inductive events, such as anterior patterning and blood island formation. Blastocyst-derived embryonic stem (ES) and trophoblast stem (TS) cell lines provide good models with which to study the development of the epiblast and trophoblast lineages, respectively. We describe the derivation and characterization of cell lines that are representative of the third lineage of the blastocyst – extra-embryonic endoderm. Extra-embryonic endoderm (XEN) cell lines can be reproducibly derived from mouse blastocysts and passaged without any evidence of senescence. XEN cells express markers typical of extra-embryonic endoderm derivatives, but not those of the epiblast or trophoblast. Chimeras generated by injection of XEN cells into blastocysts showed exclusive contribution to extra-embryonic endoderm cell types. We used female XEN cells to investigate the mechanism of X chromosome inactivation in this lineage. We observed paternally imprinted X-inactivation, consistent with observations in vivo. Based on gene expression analysis, chimera studies and imprinted X-inactivation, XEN cell lines are representative of extra-embryonic endoderm and provide a new cell culture model of an early mammalian lineage.

Key words: Primitive endoderm, X-inactivation, Chimeras, Embryonic stem cells

Introduction

Trophectoderm and primitive endoderm (PrE) are the founding cell types for two major, but distinct, extra-embryonic lineages. During mouse blastocyst formation at embryonic day 3.5 (E3.5), trophectoderm forms the outer layer of the blastocyst and by E4.5, just prior to implantation, PrE forms on the blastocoel-exposed surface of the inner cell mass (ICM) (Fig. 1A). By E5.0, the PrE is segregating into two subpopulations of extra-embryonic endoderm: visceral endoderm (VE) and parietal endoderm (PE) (Enders et al., 1978). PE cells grow with minimal cell-cell contact as they scatter on the inner surface of the giant cell layer (Fig. 1A) (Hogan and Tilly, 1981). They secrete copious amounts of basement membrane proteins to form Reichert's membrane in conjunction with the trophoblast giant cell layer (Hogan et al., 1980). The combination of PE cells, giant cells and the intervening thick basement membrane comprises the early functioning, transient parietal yolk sac (Dickson, 1979). PrE in contact with the epiblast and extra-embryonic ectoderm (trophoblast) differentiates into an epithelial sheet of visceral endoderm (Fig. 1A). VE cells in contact with extra-embryonic ectoderm are more columnar and cuboidal, while VE cells covering the epiblast are flatter and more epithelial in shape (Solter et al., 1970). They also exhibit differences in gene expression. For example, α-fetoprotein is expressed in VE overlying the epiblast (Dziadek, 1978), while *Sox7* and *Cited1* are abundant in VE overlying extra-embryonic ectoderm (Dunwoodie et al., 1998; Kanai-Azuma et al., 2002). Distal VE cells at E5.5 move asymmetrically to the future anterior region of the embryo and become anterior VE (AVE) (Thomas et al., 1998; Srinivas et al., 2004). This subset of cells appears thicker during their anterior movement and they specifically express genes not present in other parts of the VE, such as *Hex*, *Cer1* and *Otx2* (Ang et al., 1994; Biben et al., 1998; Thomas et al., 1998). The AVE induces anterior character on the overlying ectoderm by repressing posterior fates (Thomas and Beddington, 1996; Yamamoto et al., 2004). By E8.0, extra-embryonic mesoderm has become closely associated with VE to form the visceral (definitive) yolk sac. In this tissue, the VE plays an inductive role, via Indian hedgehog and VEGF, in formation of blood islands and endothelial cells (Dyer et al., 2001; Byrd et al., 2002; Damert et al., 2002).

Permanent cell lines with properties of the epiblast (embryonic stem cells) and the trophoblast (trophoblast stem cells) have been established and characterized (Evans and Kaufman, 1981; Martin, 1981; Tanaka et al., 1998). However,

reproducible derivation of cell lines with properties of the primitive endoderm lineage, including the ability to repopulate the lineage in vivo, has not been reported. The F9 embryonal carcinoma cell line has proven to be a useful model for studying VE and PE differentiation in culture (e.g. Kanungo et al., 2000; Verheijen et al., 1999b). Several other cases of cell culture models of extra-embryonic endoderm have been described previously: parietal endoderm cell (PEC) lines derived from mouse blastocysts (Fowler et al., 1990); the yolk sac carcinoma line, L2, from the rat (Wewer, 1982); and the RE1 line derived from a rat blastocyst (Notarianni and Flechon, 2001). Molecular characterization of these lines has, however, not been extensively performed, and their ability to participate in normal development remains unknown.

Described here is the characterization of cell lines representative of the extra-embryonic endoderm lineage that can be reproducibly derived from mouse blastocysts. They express a molecular profile particular to this lineage and, importantly, they are developmentally restricted to form extra-embryonic endoderm in vivo. Our first use of this novel system was to investigate the status and mechanism of X-chromosome inactivation in female XEN cells. We show XEN cells exhibit paternally imprinted X-inactivation, and that they show a novel combination of epigenetic modifications on the imprinted X, suggesting that trophoblast and extra-embryonic endoderm lineages may differ in their imprinting maintenance mechanisms.

Materials and methods

XEN cell line derivation and culture conditions

Extra-embryonic endoderm (XEN) cell lines were derived by three methods. Four lines where derived from the PO strain (Oxford University, Oxford). E3.5 blastocysts, hemizygous for the ROSA26 βgeo transgene (Zambrowicz et al., 1997), were subjected to immunosurgery to isolate their inner cell masses (ICMs) (Hogan et al., 1994; Lin, 1969). ICM pairs were aggregated and incubated overnight to obtain mini blastocysts, which were plated on embryonic fibroblasts (EMFIs) in TS cell line derivation conditions (Uy et al., 2002). The resulting blastocyst outgrowths were disaggregated 2-3 days after attachment. Although TS cell colonies were present, the vast majority of the cells were XEN cells. The XEN cell lines were designated IM5A1, IM8A1, IM8A2 and IM9C4. In the second method, XEN cell lines were derived from wild-type ICR embryos. E3.5 blastocysts were plated in four-well plates on EMFIs in RPMI 1640 (Gibco) supplemented with 20% fetal bovine serum (FBS) (CanSera, Rexdale, Canada), 1 mM sodium pyruvate, 2 mM L-glutamine, 50 mg/ml each of penicillin/streptomycin (all from Gibco), 100 μM β-mercaptoethanol (Sigma), 25 ng/ml human recombinant FGF4 (hrFGF4) and 1 μg/ml heparin (both from Sigma). The medium was changed every 3 days and the blastocyst outgrowths were not disaggregated. After 15 days, XEN cells, observed in half the cultures, were passaged 1:1 onto new EMFIs in four-well plates. Three XEN cell lines, XEN1-2, XEN1-3 and XEN1-4, derived in this manner, were routinely cultured on EMFIs in medium without FGF4 and heparin or on gelatin (0.1% porcine skin gelatin, Sigma) supplemented with 70% EMFI-conditioned medium (EMFI-CM) (Tanaka et al., 1998). A third series of XEN lines were isolated from Tgn (X^{GFP}) 4 Nagy × 129Hprt^{bm1}Pgk1a blastocysts using standard LIF-containing GMEM medium (Gibco) without FGF4 (Morey et al., 2004). Three XEN cell lines were obtained by this method: GHP7/3. GHP7/7 and GHP7/9. Selection was performed in HAT medium (100 μM hypoxanthine, 0.4 μM aminopterin, 16 μM thymidine) and 6-thioguanine (10 μg/ml). Of the various culture conditions described, we found XEN cells were the most robust in 70% EMFI-CM on gelatin and most experiments were performed under these conditions. Cells were routinely fed every 2 days and passaged at 1:10 to 1:20 every 4 days. Each XEN cell line has been cryopreserved and recovered with high viability. XEN cells were differentiated by plating them directly onto cell culture plastic, in the absence of gelatin, and culturing for 4 or 8 days in supplemented RPMI 1640 medium (described above) without hrFGF4, heparin or EMFI-CM.

ES and TS cell cultures

HP3.10 female ES cell line have been described previously (Clerc and Avner, 1998). F3 trophoblast stem cell line was isolated from F1 129/Sv Hprt-4 Pgk1a × 129/Sv embryos using a published protocol (Tanaka et al., 1998) and maintained in RPMI 1640 (Gibco) with 20% FBS (Gibco), 1 mM sodium pyruvate, 2 mM L-glutamine, 50 mg/ml each of penicillin/streptomycin (all from Gibco), 100 μM β-mercaptoethanol (Sigma), 25 ng/ml hrFGF4 and 1 μg/ml heparin (both from Sigma).

DNA transduction of XEN cells

IM8A1 cells were co-electroporated with pCX-EGFP and pPGK-puro plasmids. IM8A1 cells (5×10^6 cells at passage 39) were resuspended in PBS (0.8 ml) and transferred to a GenePulser Cuvette (0.4 cm electrode, BioRad catalog number 165-2088). *Sca*I-linearized pCX-EGFP plasmid (18 μg) (Hadjantonakis et al., 1998) and *Eco*RI-linearized pPGK-Puro plasmid (2 μg) were added to the cell suspension and electroporated (0.25 V, 500 μF). The cells were incubated on ice (20 minutes) and plated in 70% EMFI-CM (10 ml) on a gelatinized 100 mm plate. Drug selection (puromycin, 1 μg/ml) was started 1 day later, and at 12 days the colonies were passaged and expanded as a pool before FACS-sorting for GFP-positive cells (see below).

FACS analysis and subclonal lines

GFP-IM8A1 cells at one passage after electroporation (see above) were FACS-sorted for GFP fluorescence with an argon ion laser (488 nm). Approximately 800,000 GFP-positive cells were sorted into 70% EMFI-CM + G418 (90 μg/ml) + Puro (1 μg/ml) medium and plated on 0.1% gelatin in a 60 mm dish. To derive subclonal lines, 192 GFP-positive cells were single-cell sorted directly into 96-well plates.

PCR sexing

XEN cells (per single well of a four-well plate) were lysed in 100 μl of 0.1 mg/ml Proteinase K (Boehringer Mannheim) in a solution of 50 mM KCl, 10 mM Tris.HCl (pH 8.3), 2 mM $MgCl_2$, 0.1 mg/ml gelatin (Sigma), 0.45% Nonidet P-40, and 0.45% Tween-20. PCR genotyping was performed for the X-chromosome-specific *Xist* gene and Y chromosome-specific *Zfy1* gene. The primers are Xist (5′-TTG-CGGGATTCGCCTTGATT-3′) and (5′-TGAGCAGCCCTTAAAGC-CAC-3′); Zfy1 (5′-GCATAGACATGTCTTAACATCTGTCC-3′) and (5′-CCTATTGCATGGACAGCAGCTTATG-3′). PCR was performed at an annealing temperature of 65°C in 1.5 mM $MgCl_2$ for 35 cycles. The first four cycles had a 4 minute melting time at 95°C, while the next 31 cycles had a 1 minute melting time. The predicted Xist PCR product is 207 bp and Zfy1 is 183 bp. The IM5A1, IM9C4, GHP7/3 and GHP7/9 lines were genotyped as female and the XEN1-3 and GHP7/7 lines were genotyped as male (Table 1).

Videomicroscopy

XEN cells (GFP-IM8A1, subclone 1) at passage 50 were cultured on a gelatinized 100 mm plate in 70% EMFI-CM (50 ml). The microscope chamber was maintained at 37°C and 5% CO_2. Phase-contrast images were taken every 2 minutes for 12 h at 200× magnification. The imaging application used was Simple PCI by Compix (Cranberry Township, PA). The behavior of 25 XEN cells was traced for 9 out of the 12 hours. False-color (red and blue) was added to two cells using Adobe Photoshop 6.0 in 51 frames (100

minutes) of footage and the final online movie was generated with iMovie 3.

Scanning electron microscopy

XEN cells (IM8A1 at passage 29) were plated on round, plastic Thermanox coverslips (Nunc, Mississauga, Canada) in four-well plates in 70% EMFI-CM and cultured for 2 days. The cells were washed with PBS and fixed in 2% glutaraldehyde in 0.1 M phosphate buffer at 4°C overnight. The specimens were then post-fixed in 1% osmium tetroxide in the same buffer, rinsed with 0.1 M phosphate buffer and dehydrated in a graded ethanol series. The coverslips were then critical-point dried in a Bal-Tec CPD 030, mounted on aluminum stubs and sputter coated with gold in a Denton Desk II. Imaging was carried out on an FEI XL30 ESEM under standard high vacuum conditions. Most images were taken at 0° (perpendicular to the mounting stage) and some were taken at a 60° angle.

RT-PCR analysis

Total RNA was isolated from XEN cells, ES cells and embryoid bodies using the Qiagen RNeasy midi kit (Qiagen, Santa Clarita, CA) according to manufacturer's instructions. cDNA was prepared by annealing a dT_{12-18} primer (1.0 μl of 0.5 μg/μl) to 460 ng (11 μl) of total RNA at 65°C for 5 minutes. 5× RT buffer (4 μl, Invitrogen), 0.1 M DTT (2 μl), 10 mM dNTPs (1 μl) and Superscript II reverse transcriptase (1 μl, Invitrogen) were added and incubated at 42°C for 1 hour. The resulting cDNA was analyzed for the following markers: *Afp*, *Gata4*, *Sox7*, *Hnf4*, *Foxa2*, *Oct4* and β-actin (see Table S1 in the supplementary material).

Affymetrix analysis

Three biological replicates of XEN cell RNA were submitted to the Centre for Applied Genomics at the Hospital for Sick Children (Toronto, Canada) for preparation of cRNA and hybridization to the mouse U74Av2 Affymetrix gene array (Liu et al., 2003). Qiagen RNeasy midi kit (Qiagen, Santa Clarita, CA) was used to extract total RNA from all samples according to manufacturer's instructions. The samples were: (1) XEN1-3 cells at passage 18 (ICR strain, male) cultured on gelatin with 70% EMFI-CM; (2) IM8A1 cells at passage 27 (PO strain) cultured on gelatin with 70% EMFI-CM; and (3) IM8A1 cells at passage 27 cultured on tissue culture plastic in RPMI 1640 (Gibco) supplemented with 20% FBS (CanSera, Rexdale, Canada), 1 mM sodium pyruvate, 2 mM L-glutamine, 50 mg/ml each of penicillin/streptomycin (all from Gibco), 100 μM β-mercaptoethanol (Sigma) for 4 days. RNA was also obtained from R1 ES cells grown in the absence of EMFIs in standard conditions (Nagy et al., 1993) and subjected to the above analysis. A threshold significance value of $P<0.04$ was used to consider a gene expressed or 'present' and $P>0.06$ for genes not expressed or 'absent' and marginal values were $0.04<P<0.06$. Ratio data were obtained using Affymetrix MAS 5.0 software. GEO Accession Number is GSE2204.

Production of XEN cell chimeras

GFP-IM8A1 cells from a GFP-sorted population (passage 43) and from a subclonal line (GFP-IM8A1-4, passage 46) were injected into E3.5 ICR blastocysts. Between 10 and 15 XEN cells were injected per embryo and transferred to the uterus of E2.5 pseudopregnant ICR females. Dissections were performed at E6.5, E7.5 and E8.5 with special attention taken to keep the parietal yolk sac intact. X-gal staining of embryos was performed as previously described (Rossant et al., 1991). Experimental animals were treated according to guidelines approved by the Canadian Council for Animal Care.

Allelic quantitative real-time RT-PCR

Quantitative real-time PCR measurements of *Xist* cDNA were carried out using TaqMan fluorescent probes and TaqMan Universal PCR Mix and an ABI Prism 7700 (Perkin Elmer Applied Biosystem) as previously described (Morey et al., 2001). *Xist* cDNA quantitations were internally standardized against the endogenous *18s* rDNA gene. Quantitative real-time PCR measurements of *Nap1l2* cDNA were performed using SYBR Green Universal Mix. Two informative SNPs identified between the 129 and Pgk alleles were used to design allele-specific primers: Nap1l2-129-F (5′-GCACCGTCTTTTATCCC-CACT-3′), which is specific for the 129 allele; and Nap1L2-Pgk-F (5′-GCACCATCTTTTATCCCCACG-3′), which is specific for the Pgk allele. Both were used in conjunction with a universal reverse primer: Nap1L2-129/Pgk-R (5′-ACAGCAGATGCGCGATGAT-3′). *Nap1L2* cDNA quantitations were standardized against *Rrm2* cDNA (Morey et al., 2004).

RNA FISH and immunostaining

Primary antibodies used have recently been described (Okamoto et al., 2004): rabbit polyclonals detecting H3 acK9, H3 di-meK4, H3 di-meK9 (Upstate biotechnology); mouse monoclonal anti-H3 di/tri-meK27 (7B11); rabbit polyclonal anti-Enx1 (Ezh2); and mouse monoclonal anti-Eed (Sewalt et al., 1998; Hamer et al., 2002). TS and XEN cells cultured on gelatin-coated coverslips were fixed in 3% paraformaldehyde for 15 minutes at room temperature. Permeabilization was performed on ice in PBS containing 0.5% Triton X-100 and 2 mM Vanadyl Ribonucleoside Complex (VRC, Biolabs) for 3.5 minutes. After rinsing in PBS, preparations were blocked in 1% BSA (Gibco) and 0.4 U/ml RNAguard (Amersham/Pharmacia) in PBS for 15 minutes, incubated with primary antibody (diluted in blocking buffer) for 40 minutes, then washed in PBS four times, 5 minutes each, and incubated with secondary antibody (Alexa Fluor 568 goat anti-rabbit or anti-mouse, Molecular Probes) in blocking buffer for 40 minutes at room temperature. After washing in PBS, preparations were post-fixed in 3% paraformaldehyde for 10 minutes at room temperature and rinsed in 2×SSC. For RNA FISH, the *Xist* probe used was a 19 kb genomic fragment derived from a lambda clone (510) that covers most of the *Xist* gene. The probe was labeled by nick translation (Vysis) with spectrum green-dUTP (Vysis), and was hybridized (0.1 mg of probe with 10 mg of salmon sperm DNA per coverslip) in 50% formamide, 2×SSC, 20% dextran sulfate, 1 mg/ml BSA (NEB), 200 mM VRC, overnight at 37°C. After three washes in 50% formamide/2×SSC and three washes in 2×SSC at 42°C, DNA was counterstained for 2 minutes in 0.2 mg/ml DAPI, followed by a final wash in 2×SSC. Samples were mounted in 90% glycerol, 0.1×PBS, 0.1% p-phenylenediamine (Aldrich) (pH 9). A Leica DMR fluorescence microscope with a Cool SNAP fx camera (Photometrics) and Metamorph software (Roper) were used for image acquisition.

Results

Derivation of XEN cell lines

Extra-embryonic endoderm (XEN) cell lines were derived by three different methods in three different laboratories (Table 1). In the first, four XEN cell lines (IM5A1, IM8A1, IM8A2 and IM9C4) were derived from inner cell masses (ICMs) under TS cell derivation conditions (Tanaka et al., 1998). Three additional XEN cell lines (XEN1-2, XEN1-3 and XEN1-4) were derived from wild-type outbred ICR embryos by plating out single blastocysts in the aforementioned conditions. Although FGF4 was used during the derivation process of the above cell lines, this factor is not required for the maintenance of XEN cell cultures and all XEN cell lines grow well in EMFI-conditioned medium. Moreover, the presence of exogenous FGF4 is not essential during the derivation process because a third series of XEN cell lines, GHP7/3, GHP7/9 and GHP7/7, was isolated from E3.5 blastocysts using standard LIF-containing medium without FGF4. Six XEN cell lines have

Table 1. XEN cell lines

XEN cell line	Mouse strain/genotype	Sex	Laboratory
GHP7/3	Tgn (X^{GFP})4 × 129Hprt^{bm1}Pgk1a	F	Avner
GHP7/7	Tgn (X^{GFP})4 × 129Hprt^{bm1}Pgk1a	M	Avner
GHP7/9	Tgn (X^{GFP})4 × 129Hprt^{bm1}Pgk1a	F	Avner
IM5A1	PO strain; ROSA26 βgeo/+	F	Gardner
IM8A1	PO strain; ROSA26 βgeo/+	–	Gardner
IM8A2	PO strain; ROSA26 βgeo/+	–	Gardner
IM9C4	PO strain; ROSA26 βgeo/+	F	Gardner
XEN1-2	ICR strain; wild type	–	Rossant
XEN1-3	ICR strain; wild type	M	Rossant
XEN1-4	ICR strain; wild type	–	Rossant

Summary of the 10 XEN cell lines, indicating their background, sex (where known) and the laboratory where it was derived.

been passaged at least 12 times each without undergoing senescence or a reduction in viability, with one cell line (IM8A1) being passaged more than 50 times (more than 6 months in culture). All XEN cell lines have been cryopreserved and recovered without loss of viability. The sex of two PO XEN cell lines, one ICR XEN cell line and all three of the GHP XEN cell lines was determined by X and Y chromosome-specific PCR. Two of the lines are male (XY: XEN1-3 and GHP7/7) and four are female (XX: IM5A1, IM9C4, GHP7/3 and GHP7/9), demonstrating that there is no restriction on the sex of XEN cells. FACS analysis with propidium iodide determined that the vast majority of XEN cells contain a diploid DNA content with some tetraploid cells. Two GHP XEN cell lines were further characterized for their karyotype, which confirmed the majority of cells were diploid (data not shown). The extent to which these cell lines are chromosomally stable and remain euploid during prolonged culture awaits detailed investigation.

In summary, XEN cell lines can be consistently derived from blastocysts or ICMs from different genetic backgrounds. Derivation from ICMs was more efficient than from intact blastocysts. However, once derived all XEN cell lines could be maintained in EMFI-CM on gelatin without supplementation with additional growth factors.

XEN cell morphology and behavior

In contrast to the coherent colonies formed by ES and TS cells, XEN cells maintain very little cell-cell contact when grown at low density. The cultures contain at least two cell morphologies; a rounded, highly refractile cell type and a more stellate epithelial-like cell type (Fig. 1B). At higher densities XEN cells could form epithelial sheets (Fig. 1C) and often formed a lattice-type structure (Fig. 1D). When XEN cells were removed from gelatin and plated on tissue culture plastic without EMFI-CM, many cells became large and vacuolated within 6 days (Fig. 1E) and the cell lines could no longer be passaged. Although there was some variability observed between different cell lines in terms of proliferation rates and ratios of round versus epithelioid cell types, the basic morphology of the cultures was consistent and easily recognizable.

To investigate whether the presence of the two cell morphologies represented two cell types in the starting cultures, we FACS-sorted single GFP-labeled XEN cells directly into 96-well plates and subclonal cell lines were derived. Cell viability was ~50% and 16% (31/192) of the subclonal cultures could be passaged. Importantly, all subclonal lines exhibited both round and epithelial-like cell morphologies. This suggested that the two cell morphologies observed in the parental cell line was not due to a mixture of

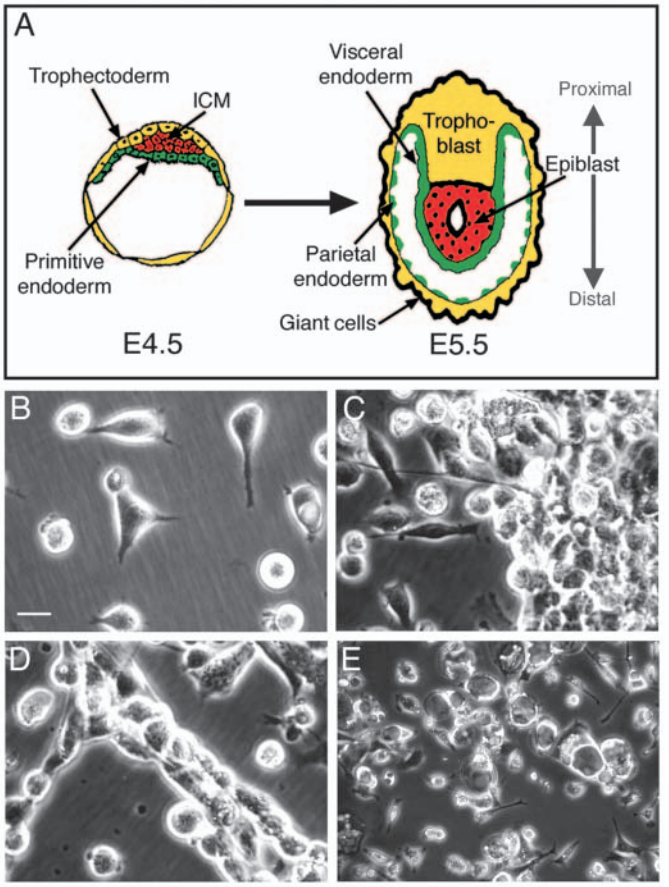

Fig. 1. The extra-embryonic endoderm lineage and XEN cell cultures. (A) Schematic of an E4.5 blastocyst and an E5.5 embryo, illustrating the primitive, visceral and parietal endoderm (green). The trophoblast and epiblast lineages are represented in yellow and red, respectively. The proximodistal axis is indicated. ICM, inner cell mass. (B) Phase-contrast micrograph of XEN cells cultured at low density illustrating the refractile, rounded cell type and the epithelioid cell type. (C) XEN cells grown to near confluency showing the presence of individual cells and an epithelial sheet of cells. (D) XEN cells participating in a lattice-type structure. (E) XEN cells plated in the absence of gelatin resulted in differentiation of some cells into large vacuolated cells in 6 days. Scale bar: 10 μm for B-D; 40 μm for E.

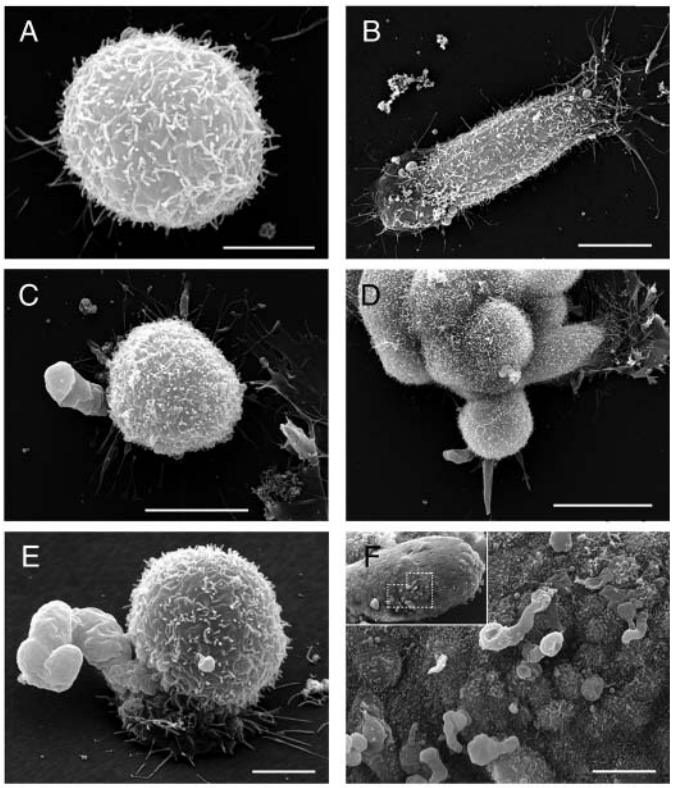

Fig. 2. Scanning electron microscopy of XEN cells and a pre-gastrula embryo. (A) Rounded XEN cell with numerous microvilli. (B) Epithelial-like XEN cell with numerous microvilli and lamellepodia at opposite ends. (C) Isolated XEN cell with a short pseudopodium. (D) Colony of XEN cells where one cell is extending several pseudopodia. (E) Isolated XEN cell with a curving pseudopodium viewed at a 60° angle. This XEN cell is on a pedestal structure and the pseudopodium is originating from below the cell. (F) E5.5 embryo with Reichert's membrane removed (inset) and a higher magnification of the anterior visceral endoderm region (broken line). Scale bars: 5 μm for A,E; 10 μm for B,C,F; 20 μm for D.

two distinct cell populations that were being co-cultured. Videomicroscopy showed that XEN cells are highly motile and that single cells made transitions between round and epithelioid cell types without cell division (see Movie 1 in the supplementary material). Twenty-five individual XEN cells were followed for a 9-hour period by videomicroscopy and 76% (19/25) underwent reversible phenotypic changes (round-to-epithelioid-to-round or epithelioid-round-epithelioid) without cell division. Thus, the two cell morphologies represent different phases of the dynamic behavior of XEN cells in culture.

During these studies, we observed a large, unusual cell process being transiently produced by 25% of XEN cells in their rounded, but not their epithelioid, phase. The cellular process was rapidly extruded and retracted (within 6-10 minutes) and was often longer than a cell diameter and as wide as 30-40% of the thickness of the cell. Scanning electronmicroscopy (SEM) revealed that although both the rounded and epithelioid XEN cell surfaces were very dense with microvilli (Fig. 2A,B), the large pseudopodium was completely devoid of them (Fig. 2C-E). SEM images taken at a 60° angle showed that the pseudopodia were not attached to the cell culture substrate, but were raised above it (Fig. 2E). PE and VE of E5.0-E6.5 embryos were also analyzed by SEM and very similar pseudopodia were found protruding from the distal and anterior VE regions of E5.25-E5.75 embryos (Fig. 2F). These protrusions were not observed on PE cells or other regions of the VE, and disappeared completely by E6.5. Thus, although the overall morphology of the XEN cultures resembles PE, the presence of pseudopodia is a property more typical of VE.

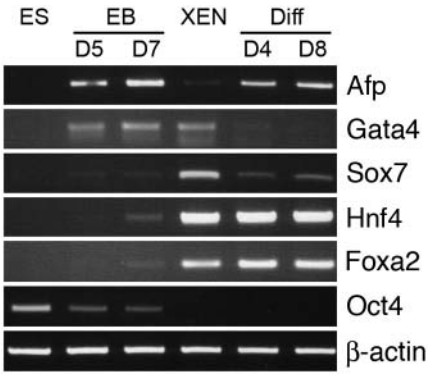

Fig. 3. RT-PCR expression analysis of XEN cells. cDNA was prepared from ES cells, embryoid bodies (EB) at day 5 (D5) and day 7 (D7), and three different XEN cell cultures. XEN cells were either cultured on gelatin in maintenance conditions (XEN) or differentiated (Diff) by removal of EMFI-CM and gelatin for 4 or 8 days (D4, D8). *Hnf4* and *Foxa2* were consistently detected in all XEN cell cultures, while *Gata4* and *Sox7* decreased expression upon differentiation. By contrast, α-fetoprotein (*Afp*) increased with differentiation. *Oct4* was undetectable in XEN cells.

XEN cells express markers of extra-embryonic endoderm

We performed an initial expression analysis of XEN cells by reverse-transcriptase-PCR (RT-PCR) on RNA isolated from XEN cells cultured under maintenance and differentiation conditions. We investigated several known markers of the extra-embryonic endoderm. The VE marker α-fetoprotein (*Afp*) was barely detectable in maintenance cultures, but increased upon differentiation. By contrast, *Gata4* and *Sox7* expression decreased upon differentiation, while *Hnf4* and *Foxa2* levels remained constant (Fig. 3).

XEN cell lines were further characterized by microarray gene expression analysis of three biological replicates. In order to identify genes that were consistently expressed, irrespective of background or culture medium, the replicates were chosen to provide samples from different mouse strains under different culture conditions. The XEN cell cultures were: (1) XEN1-3 cells (ICR strain, passage 18) grown on gelatin with EMFI-CM (sample 'XEN1-3′); (2) IM8A1 cells (PO strain, passage 27) cultured as above (sample 'IM8A1-I'); and (3) IM8A1 cells (passage 27) grown on tissue culture plastic without EMFI-CM for 4 days (sample 'IM8A1-II'). The third sample (IM8A1-II), grown in differentiation conditions, exhibited some morphological changes, such as larger cells, but vacuolated cells were not present. The mouse U74Av2 Affymetrix array was used for this analysis. A fourth sample, undifferentiated

ES cells, was also analyzed on the same array. Data were analyzed for expression of specific marker genes of visceral and parietal endoderm and genes characteristic of ES and TS cells (Table 2). Signaling pathways, selected gene families and transcription factors involved in early development were also analyzed in this manner (see Table S2 in the supplementary material).

Genes specifically expressed in parietal endoderm alone, and not in visceral endoderm or markers common to both lineages were highly expressed in XEN cells (Table 2). Extracellular matrix (ECM) proteins expressed by parietal endoderm to make Reichert's membrane were also abundant. Perlecan (heparin sulfate proteoglycan 2), type IV collagen (α1 and α2 chains only), *laminin* (α1, β1, and β2 chains), and nidogens 1 and *2* (Hogan et al., 1980; Semoff et al., 1982) were expressed in all XEN cell samples (see Table S2 in the supplementary material).

Several genes implicated, by mutant analysis, in visceral endoderm function were also expressed in XEN cells. These genes are, however, are also expressed in primitive and parietal endoderm and may be more representative of primitive endoderm. They include *Gata4* and *Gata6* (Arceci et al., 1993; Koutsourakis et al., 1999; Narita et al., 1997), *Disabled2* (Morris et al., 2002; Yang et al., 2002), *Tcf2* (*vHnf1*) (Barbacci et al., 1999) and *Vegfa* (Miquerol et al., 1999). Markers specific to VE are often difficult to identify because of the lack of comparable expression data for parietal endoderm. From published expression data where both PE and VE were investigated, several VE-specific genes have been identified. A number of these genes were expressed in XEN cells and they include *Foxa2* (Dufort et al., 1998), *Ihh* (Becker et al., 1997) and type I Acvr1 (Gu et al., 1999). Other VE-specific genes, such as *Afp*, *Hnf4* and *uPA* (*Plau*) were not detected (Table 2). However, *Afp* can be induced in differentiated XEN cell

Table 2. Expression of known genes in XEN cells

Affy ID	Unigene	Gene name or symbol	XEN1-3	IM8A1-I	IM8A1-II	Reference
PE genes						
100717_at	Mm.2093	*Snail*	590	660	239	Veltmaat et al., 2000
95079_at	Mm.221403	*Pdgfra*	1057	1487	1056	Mercola et al., 1990
104601_at	Mm.24096	Thrombomodulin	219	393	199	Healy et al., 1995
93981_at	Mm.154660	tPA, tissue-type plasminogen activator	1641	1893	1191	Marotti et al., 1982
98482_at	Mm.3542	*Pthr1*	2276	2128	1384	Verheijen et al., 1999a
98817_at	Mm.4913	Follistatin	1869	3475	2990	Feijen et al., 1994
97160_at	Mm.291442	*Sparc*	5924	7284	7376	Mason et al., 1986
VE genes						
93950_at	Mm.938	*Foxa2* (Hnf3β)	552	512	520	Dufort et al., 1998
160705_at	Mm.2390	*Cited1* (Msg1)	1208	1032	1651	Dunwoodie et al., 1998
103949_at	Mm.2543	Indian hedgehog (*Ihh*)	182	63	156	Becker et al., 1997
98320_at	Mm.42230	*Cyp26a1* (*P450RAI*)	354	1721	1010	Fujii et al., 1997
100515_at	Mm.5241	*Furin*	450	327	455	Roebroek et al., 1998
93460_at	Mm.689	*Acvr1*, Activin A receptor, type 1	93	65	64	Gu et al., 1999
92713_at	Mm.202383	*Hnf4*	A	A	A	Duncan et al., 1994
101493_at	Mm.358570	*Afp*, α-fetoprotein	A	A	A	Dziadek and Adamson, 1978
101494_at	Mm.358570	*Afp*, α-fetoprotein	A	A	A	Dziadek and Adamson, 1978
97772_at	Mm.4183	*Plau*, plasminogen activator, urokinase	A	A	A	Marotti et al., 1981
PE and VE genes						
102713_at	Mm.247669	*Gata4*	1091	1196	899	Arceci et al., 1993
104698_at	Mm.329287	*Gata6*	2468	1973	1086	Koutsourakis et al., 1999
92487_at	Mm.42162	*Sox7*	981	1339	225	Kanai-Azuma et al., 2002
92997_g_at	Mm.279103	*Sox17*	1024	1436	1285	Kanai-Azuma et al., 2002
92996_at	Mm.279103	*Sox17*	1293	1895	1712	Kanai-Azuma et al., 2002
101396_at	Mm.7226	*Dab2*, *Tcf2* (*vHnf1*)	111	189	138	Barbacci et al., 1999
98044_at	Mm.240830	Disabled2	206	277	624	Yang et al., 2002
102258_at	Mm.10801	*Stra6*	465	296	85	Bouillet at al., 1997
103520_at	Mm.282184	*Vegfa*	112	263	158	Miquerol et al., 1999
101009_at	Mm.358618	Krt2-8 (Endo A)	1358	1962	2737	Hashido et al., 1991
94270_at	Mm.22479	Krt1-18 (Endo B)	1192	2104	3834	Oshima, 1981
ES and TS cell genes						
103075_at	Mm.17031	*Pou5f1* (*Oct4*)	A	A	A	Palmieri et al., 1994
161072_at	Mm.6047	*Nanog*	A	A	A	Wang et al., 2003
98414_at	Mm.285848	*Zfp42* (Rex1)	A	A	A	Rogers et al., 1991
93880_at	Mm.200692	*Eomes*, Eomesodermin	A	A	A	Tanaka et al., 1998
103532_at	Mm.200692	*Eomes*, Eomesodermin	A	A	A	Tanaka et al., 1998
100301_at	Mm.235550	*Esrrb*, Errβ	A	A	A	Tanaka et al., 1998
103239_at	Mm.20358	*Cdx2*	A	A	A	Tanaka et al., 1998

A subset of genes from expression analysis of XEN cells by Affymetrix profiling (from the mouse U74Av2 Affymetrix array). Data are shown for genes known to be expressed in the VE, PE and other embryonic lineages. The Affy Clone ID, Unigene designation and gene name or symbol are listed. The three XEN samples (XEN1-3, IM8A1-I and IM8A1-II) are described in the text. If a gene is expressed at statistically significant levels ($P<0.04$), its expression level is indicated by a numerical value; if a gene is not expressed, it is indicated by 'A' (absent). A single reference is provided for each gene and others are mentioned in the text. The groupings are a rough guide, as PE expression data for many genes are not available. In addition, many of these genes are expressed at other stages and tissues during development.

Table 3. XEN cell chimera data

XEN cells	Stage	Number transferred	Number of embryos	Number of chimeras
Population	E6.5	34	30	8
	E7.5	36	23	8
	E8.5	42	25	7
Clone 4	E6.5	42	27	13
	E7.5	42	30	10
	E8.5	41	33	4
Total	E6.5-E8.5	237	168 (71%)	50 (30%)

GFP-IM8A1 XEN cells (FACS-sorted population) and a subclonal line, GFP-IM8A1-4 (Clone 4), were used to generate chimeras by blastocyst injection. The stage dissected, the number of blastocysts transferred, the number of embryos recovered and the number of chimeras observed are indicated.

cultures and *Hnf4* is indeed detectable by RT-PCR (Fig. 3). Genes representative of TS cells, *Cdx2*, *Eomes* and *Esrrb* (Tanaka et al., 1998), and the ES cell markers, *Oct4*, *Nanog* and *Rex1*, were not detected in XEN cells (Table 2). We conclude, based on our marker studies, that XEN cells derive from the primitive endoderm of the blastocyst and represent derivatives of this lineage.

As well as examining the expression of known extra-embryonic endoderm markers, we also used a comparison of ES cell and XEN cell Affymetrix expression data to seek those genes specifically enriched in XEN cells. The three XEN cell data sets described above were each compared with a single data set from undifferentiated ES cells and three expression ratios were obtained for each gene. These ratios were averaged and rank-ordered, starting with the largest ratio. Within the list of 40 genes with the highest positive expression ratios, at least 13 genes are known extra-embryonic endoderm markers (see Table S3 in the supplementary material). This was in contrast to the list of genes with the largest negative expression ratios, which was highly enriched with known ES cell-specific genes (see Table S4 in the supplementary material).

XEN cell chimeras

We investigated the developmental potential of XEN cells by generating chimeras with a GFP/*lacZ* cell line and one of its subclonal derivatives (Table 3). XEN cells (10-15) were injected into wild-type ICR blastocysts and analyzed for contributions at E6.5, E7.5 and E8.5. GFP/*lacZ* XEN cells were never found to contribute to the embryo proper, yolk sac mesoderm or the trophoblast lineage. The overwhelming number of chimeras (49/50) had XEN cell contributions restricted to parietal endoderm. At E6.5, XEN cells were usually observed as dispersed cells lining the distal part of the parietal yolk sac (Fig. 4A,B). At later stages, chimeras could be found with large numbers of scattered XEN cells in their parietal yolk sacs (Fig. 4C-E). This pattern was typical of parietal endoderm growth in vivo (Hogan and Newman, 1984). A single chimera exhibited a contribution of XEN cells in the visceral endoderm layer (Fig. 4F), but not in the parietal yolk sac (not shown). The nature of this clone was very different from the contributions to the parietal region, as the XEN cells formed a coherent epithelial sheet without intermingling with host cells. This is consistent with the coherent growth characteristics of VE in vivo (Gardner and Cockroft, 1998).

Imprinted X chromosome inactivation in XEN cells

The evidence that XEN cell lines represent cells of the extra-embryonic endoderm lineage led us to analyze the X-inactivation status of female XEN cell lines. If X-inactivation has yet to occur, *Xist* expression should be low from both the maternal and paternal X chromosomes. If imprinted X-inactivation, which appears to onset very early in mouse embryogenesis (Okamoto et al., 2004), has already occurred, *Xist* expression would be expected to be associated with silencing of genes on the paternally inherited X chromosome. Our studies concentrated on the GHP7/9 and GHP7/3 cell lines that are heterozygous for the $Hprt^{bm1}$ mutation and have a maternal X-linked GFP transgene. They also carry polymorphisms for X-linked genes, such as *Xist* and *Nap1l2*. We first addressed whether an inactive X chromosome was present and whether it was subject to imprinting. We used allelic quantitative RT-PCR to assess whether *Xist* and *Nap1l2*

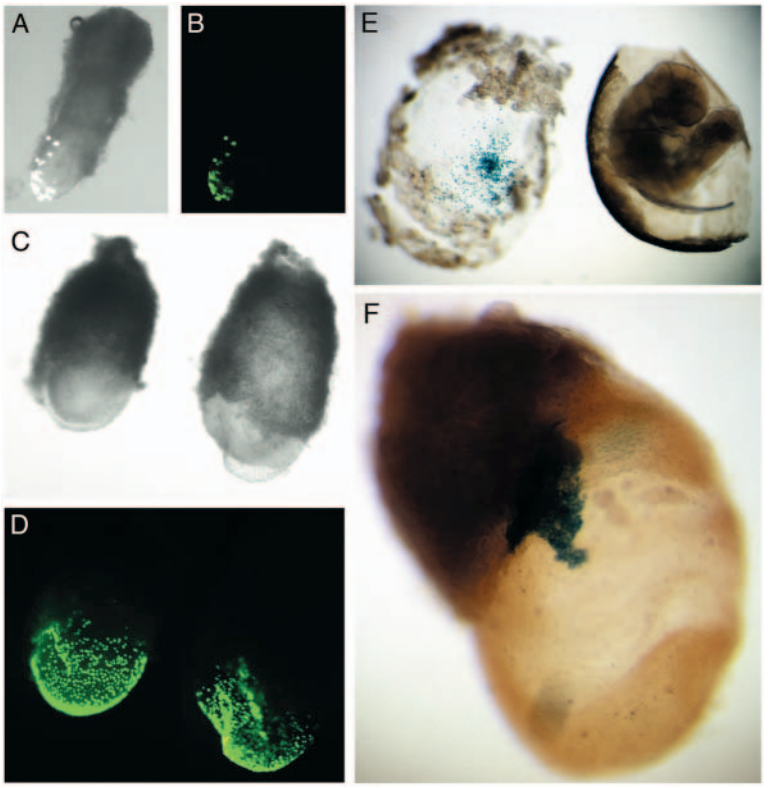

Fig. 4. XEN cell chimeras. (A,B) E6.5 chimera with XEN cells contributions to the distal region of the conceptus in the parietal yolk sac: (A) Partial phase-contrast and UV fluorescence micrograph; (B) UV fluorescence micrograph with false-color added. (C,D) Two E7.5 chimeras with XEN cell contributions exclusively to the parietal yolk sac; phase-contrast (C) and UV fluorescence micrographs (D). (E) E8.5 XEN cell chimera in which the parietal yolk sac was dissected from the visceral yolk sac and embryo proper after X-gal staining. All the X-gal-positive cells were located in the parietal yolk sac and not in other tissues. (F) E7.5 XEN cell chimera after X-gal staining. A coherent XEN cell clone was observed in the extra-embryonic region of the visceral endoderm.

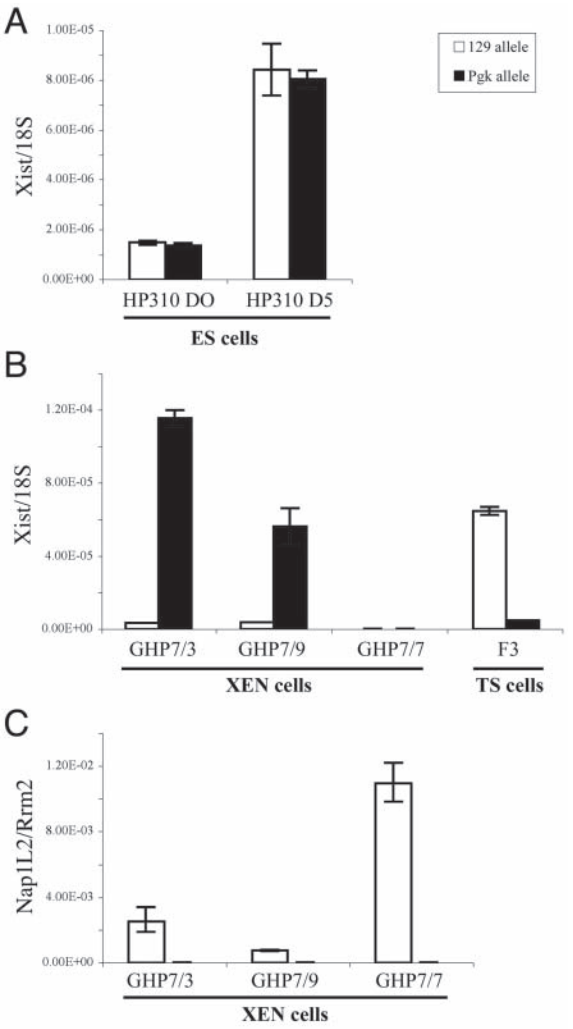

Fig. 5. X-inactivation in ES, TS and XEN cells. Quantification of *Xist* transcripts by real-time RT-PCR in (A) ES cells and (B) XEN and TS cells normalized using the endogenous gene *18S*. (C) Quantification of *Nap1l2* transcripts normalized using the *Rrm2* gene in XEN cells. In differentiated ES cells (A), random X-inactivation leads to equivalent levels of *Xist* expression from the 129 and Pgk alleles. By contrast, in female XEN cells (GHP7/3 and GHP7/9), *Xist* is expressed only from the paternal Pgk allele (B) and *Nap1l2* only from the maternal 129 allele (C), indicating that the paternal X chromosome is preferentially inactivated in female XEN cells, just as in female TS cells (where *Xist* is expressed only from the paternal 129 allele; B). In control male XEN cells (GHP7/7), the single X chromosome derived from 129 is active, as shown by the absence of *Xist* (B) and presence of *Nap1l2* expression (C).

were expressed from the maternal or the paternal X chromosome. In undifferentiated female ES cells, *Xist* expression was low and similar for both parental X chromosomes. The equal increase of maternal and paternal *Xist* expression in differentiated ES cells is indicative of random X-inactivation (Fig. 5A). By contrast, in female XEN cell lines *Xist* expression was overwhelmingly of the 'Pgk' allelic form that corresponds to the paternal allele and was absent from the male GHP7/7 XEN cell line (Fig. 5B). TS cells, harboring a '129' paternal *Xist* allele showed a similar pattern of expression. In agreement with this, uniparental *Nap1l2* expression indicated the presence of an active maternal X chromosome in XEN cells (Fig. 5C). Quantitative RT-PCR analysis of GFP expression, although variable, showed that GFP expression was at least eightfold greater in the female GHP7/3 and GHP7/9 cell lines carrying a maternally inherited X-linked GFP transgene, than in control TS cell lines carrying a paternally inherited X-linked GFP transgene (data not shown). We conclude that the paternal X chromosome expressing *Xist* was chosen to be inactivated in female XEN cell lines.

Growth of XEN cell lines on HAT and 6-thioguanine (6TG) media allows for a more global assessment of stability of the inactive X chromosome by assaying for the presence or absence of a functional *Hprt* gene. The GHP7/9 XEN cell line carrying a functional *Hprt* gene on the maternal X and a non-functional $Hprt^{bm1}$ allele on the paternal X grew on HAT medium and failed to grow on 6TG medium (data not shown). These results support the idea that the paternally inherited X chromosome carrying the mutated allele was inactivated and that the inactivation state is stable in long-term cultures.

When RNA FISH was carried out on female XEN cells, a Xist domain was found in the majority of cells analyzed (Fig. 6A). A pinpoint signal was not found to be associated with the active X chromosome and neither domain nor pinpoint Xist signals were found in the male XEN cell line (GHP7/7). Chromatin modifications associated with the inactive X chromosome in females were examined by Immuno-RNA FISH in female XEN cells. Female TS cell lines were concurrently examined to explore possible differences between the two extra-embryonic lineages. Unlike TS cells, which show an enrichment of Eed and Ezh2 (Mak et al., 2002), the Xist domain in XEN cells show no enrichment for these polycomb group proteins (compare Fig. 6A with 6B). Histone H3 dimethylated K4 (H3 di-meK4), which is normally associated with active euchromatin, is excluded from the Xist domain in both XEN and TS cells, as is histone H3 acetylated K9 (H3 acK9) (Fig. 6; see Fig. S1 in the supplementary material). Histone H3 di-meK9, which has been associated with the inactive X chromosome in differentiating ES cells and somatic cells (Heard et al., 2001), appears only weakly enriched, if at all, in XEN and TS cells (see Fig. S1 in the supplementary material). Histone H3 tri-meK27, which has recently also been associated with the inactive X in both TS cells and differentiating ES cells (Silva et al., 2003), is enriched over the Xist domain in only a small proportion of XEN cells. This is consistent with the absence or low levels of Ezh2 on the X chromosome in XEN cells, as Ezh2 is thought to be the histone methyltransferase responsible for the H3 tri-meK27 mark (Erhardt et al., 2003). Thus, XEN cells exhibit a unique combination of histone modifications on their inactive X chromosome – which includes hypoacetylation of H3 K9, hypomethylation of H3 K4 and little or no methylation of H3 K27 and K9. The stability of the inactive state of the X chromosome in these cells, despite the absence of H3 K9 and H3 K27 methylation, suggests that other epigenetic marks are involved. This is different from the situation in ES cells, where the lack of Ezh2 accumulation and H3 K27 methylation results in significant reactivation of X-linked genes (Silva et al., 2003).

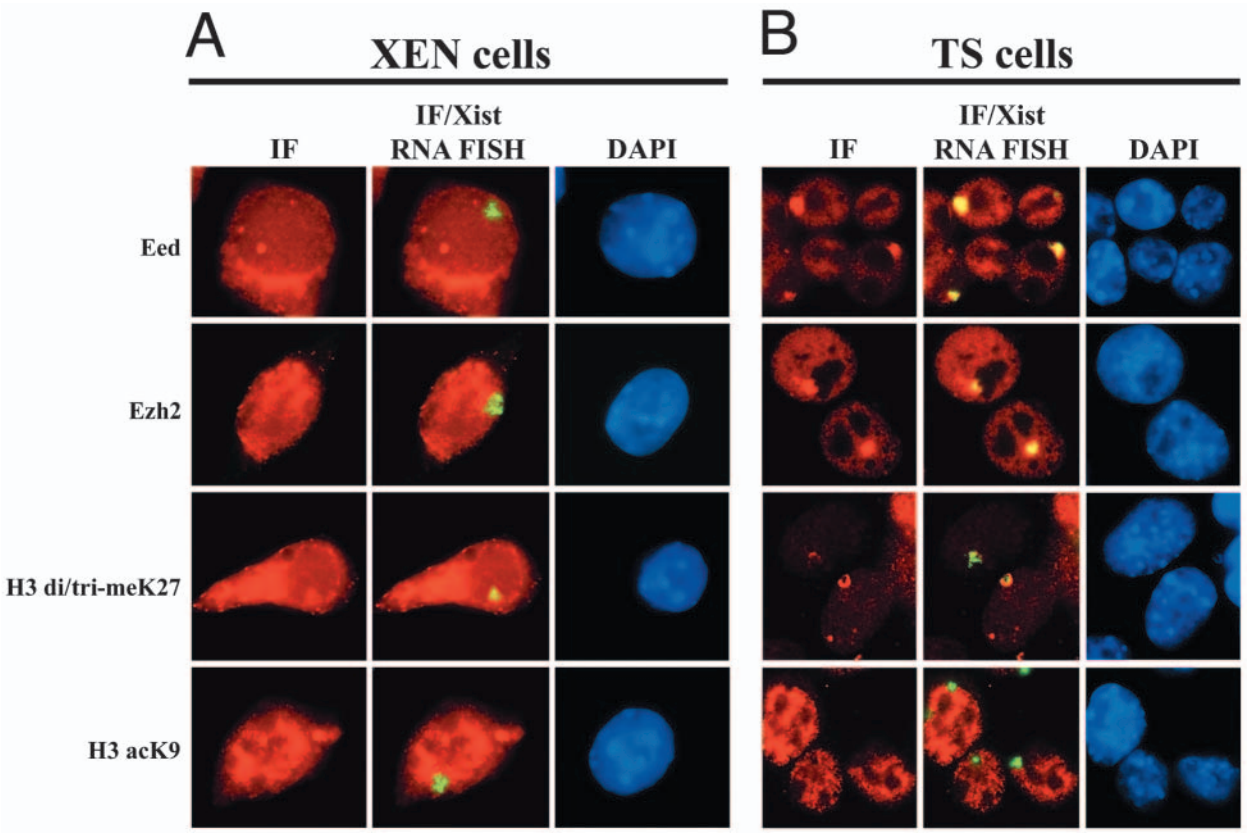

Fig. 6. Histone modifications and *Xist* accumulation in female XEN and TS cells. (A,B) Representative images show immunofluorescent detection of Eed, Ezh2 and histone modifications H3 di/tri-methyl K27 and H3 acetyl K9, combined with *Xist* RNA FISH. Immunodetections were performed using Alexa 568-conjugated secondary antibodies (red). *Xist* RNA was detected using a Spectrum Green-labeled FISH probe (green). XEN cells (A) show no Eed and Ezh2 enrichment, and weak H3 di/tri-meK27 enrichment on the *Xist*-coated X chromosome, but they do show depletion for H3 acK9. By contrast, undifferentiated TS cells (B) show enrichment for Eed, Ezh2 and H3 di/tri-meK27 on the *Xist*-coated X chromosome. Depletion for H3 acK9 was observed, as in XEN cells.

Discussion

We have derived and characterized a number of independent cell lines from mouse blastocysts that exhibit extra-embryonic endoderm characteristics. This ex vivo model, which is reproducibly derived from early embryos, is distinct from ES and TS cell lines. We show here that XEN cells exhibit mixed characteristics of parietal endoderm and visceral endoderm, and can contribute to both lineages in chimeras in vivo, although they are biased towards parietal endoderm. We also show that they exhibit paternal X-inactivation, but without the association of Eed and Ezh2 that typifies the inactive X in TS cells.

XEN cell maintenance

XEN cell cultures are robust and easily maintained, but their precise signaling requirements are not known. After derivation, they can be grown on gelatin in medium supplemented with EMFI-CM. The FGF signaling pathway required for TS cells is not important for XEN cell maintenance and indeed none of the four FGF receptors is expressed in XEN cells (see Table S2 in the supplementary material). The LIF-STAT pathway may be playing a role in XEN cell maintenance. Several components (LIFR, gp130, JAK1, JAK2, STAT1 and STAT3) of this pathway are expressed in XEN cells (see Table S2 in the supplementary material), and their derivation and maintenance on EMFIs or EMFI-CM would provide an adequate source of LIF (Smith et al., 1992).

XEN cells show properties of both parietal and visceral endoderm

The previously reported mouse PEC and rat RE1 cell lines are similar, if not identical, to the XEN cells described here. The two distinct morphologies of XEN cells in culture – rounded highly refractile and flattened epithelioid – have also been reported (Fowler et al., 1990; Notarianni and Flechon, 2001). These cell lines are considered representative of parietal endoderm based on their morphology and the secretion of ECM proteins abundantly found in Reichert's membrane. Our Affymetrix expression analysis of XEN cells supports the idea that PE properties are highly represented in such cell culture models. PE-associated genes, such as *tPA* (*Plat*), thrombomodulin, *Snail* and *Pdgfra*, were strongly expressed. Genes encoding for basement membrane proteins (perlecan, type IV collagen, laminin and nidogen), abundant in Reichert's membrane, were also expressed at high levels in XEN cells.

XEN cells, however, also expressed several VE-associated genes in all cultures (Table 2). The high expression of *Sox7*, *Sox17* and *Cited1* were of particular interest, given their

restricted expression in VE overlying the extra-embryonic ectoderm and in the marginal zone VE (Dunwoodie et al., 1998; Kanai-Azuma et al., 2002). The reduction of *Sox7* and increase of *Afp* expression in differentiated XEN cell cultures suggests that XEN cells may have some properties of extra-embryonic VE when cultured in standard conditions, but can be differentiated to cells more related to the VE overlying the epiblast in other conditions. Furthermore, *Sox7* expression suggests that definitive endoderm is not present in XEN cell cultures, as this gene, unlike *Sox17*, is not detectable in definitive endoderm in vivo (Kanai-Azuma et al., 2002).

The single-cell clonal analysis and videomicroscopy studies of XEN cells have indicated that the rounded and epithelial-like cell types are lineage-related and reversibly interchangeable. The morphology of marginal zone VE cells in vivo is similar to the flattened, epithelial-like cells observed in culture. The marginal zone cells have been described as mesenchymal with ruffled cell membranes reminiscent of lamellipodia (Hogan and Newman, 1984). The unusual pseudopodia we observed in XEN cells were also observed in the AVE region of early postimplantation embryos in vivo (Fig. 2F), suggesting that XEN cells may also transiently become AVE like. Taken together, the gene expression studies and cell morphology/behavior suggest that clonal XEN cell cultures exhibit properties of PE, marginal zone VE, AVE and extra-embryonic VE, but not of embryonic VE. However, the induction of an embryonic VE marker, *Afp*, in differentiated cultures of XEN cells (Fig. 3) suggests that this VE subtype could be induced in defined conditions.

XEN cell chimeras

XEN cell lines retain the capacity to contribute to primitive endoderm derivatives in vivo in chimeras, attesting to their primitive endoderm nature and the stability of their ex vivo phenotype. However, they exhibited a strong bias to form parietal endoderm in chimeras, with only one visceral endoderm clone observed. This does not necessarily indicate their full potential. The blastocyst injection procedure used to generate chimeras may have provided an environment that promotes PE and hinders VE differentiation. XEN cells injected into the blastocoel are likely to end up in the superficial layer of the primitive endoderm as it forms (Gardner, 1985) or associated with the TE away from the ICM. PrE or VE cells that lose contact with epiblast and extra-embryonic ectoderm default to a PE phenotype or are instructed by the trophectoderm to become PE by a combination of TE basement membrane and the PTHrP/cAMP signaling pathway (Verheijen et al., 1999a). This observation is supported by chimera experiments with primitive endoderm cells and nascent VE cells directly isolated from embryos. Blastocyst injection of PrE or early VE cells also resulted in chimeras with mostly PE contributions (Gardner, 1982; Cockroft and Gardner, 1987). Although the donor PrE and VE cells clearly have the potential to make VE, their behavior in chimeras did not reflect this. Given this intrinsic bias, an alternative method for producing chimeras, such as morula or ICM injections, may be required to observe the full potential of XEN cells.

Imprinted X-inactivation in XEN cells

The extra-embryonic trophoblast and endoderm lineages of the mouse undergo imprinted inactivation of the paternal X chromosome (Xp) (Takagi and Sasaki, 1975). Initiation of X inactivation requires expression of the non-coding RNA, *Xist*, and accumulation of this transcript on the inactive X chromosome in cis (Penny et al., 1996). In agreement with their proposed extra-embryonic endodermal origin, XEN cells maintain imprinted X inactivation of the Xp and exhibit *Xist* accumulation on one X chromosome. This Xist domain also exhibits other epigenetic marks of being inactive, such as exclusion of histone H3 acetylated K9 and dimethylated K4, both hallmarks of active euchromatin (Heard et al., 2001; Boggs et al., 2002). Surprisingly, accumulation of the polycomb group proteins, Eed and Ezh2, was not observed in XEN cells, as is seen in TS cells (Fig. 6) (Mak et al., 2002). Mutant analysis revealed an important role for *Eed* in the formation of trophoblast giant cells, but not extra-embryonic endoderm, during development (Wang et al., 2002). The proposed substrate of Ezh2, histone H3 K27, appears to be weakly di/trimethylated in some XEN cells, suggesting a transient association of Ezh2. Thus, the mechanisms to maintain X inactivation in XEN cells, TS cells and ES cells differ at the levels of polycomb group proteins and histone modifications. The HAT/6TG studies indicate that the inactive state of the Xp in XEN cells is stable, despite the absence of H3 meK9 enrichment and low levels of H3 K27 methylation. This suggests XEN cells may have different mechanisms for maintenance of their inactive X chromosome and that other epigenetics marks likely account for the stability of the inactive state. These results illustrate the cell lineage-dependent variation in mechanisms of X-inactivation maintenance that has also been observed in other systems (Plath et al., 2003).

XEN cells from ES cells

The XEN cell lines described here were derived de novo from blastocysts. However, two reports suggest that similar cell lines can be derived directly from established ES cell lines. Overexpression of the GATA factors, GATA4 or GATA6, in ES cells induced uniform differentiation into extra-embryonic endoderm cells (Fujikura et al., 2002). In terms of marker analysis and cell morphology, these cells appear identical to XEN cells. Fujikura et al., hypothesize that a repressor of *Gata4/6* expression is required to maintain ES cells pluripotent. The recently identified pluripotent transcription factor, Nanog, may fulfill this role (Chambers et al., 2003; Mitsui et al., 2003). *Nanog*$^{-/-}$ ES cells express many markers of extra-embryonic endoderm (*Gata6*, *Tcf2* and *Ihh*) and are also morphologically very similar to XEN cells (Mitsui et al., 2003). This implicates Nanog as a general repressor of the extra-embryonic endoderm lineage and it may function, in part, through repression of key regulatory genes, such as *Gata6*.

XEN cell lines provide a unique model for an early mammalian lineage that will complement the established ES and TS cell lines. Through the study of essential genes and signaling requirements for this cell culture system, insights will be gained about the developmental program of the extra-embryonic endoderm lineage. In addition, in vitro combinations of ES, TS and XEN cells may help model the in vivo interactions between embryonic and extra-embryonic lineages important for embryonic patterning.

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Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/132/7/1649/DC1

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