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Cyclins E1 and E2 are required for endoreplication in placental trophoblast giant cells

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In mammalian cells, cyclin E–CDK2 complexes are activated in the late G_1 phase of the cell cycle and are believed to have an essential role in promoting S-phase entry. We have targeted the murine genes *CCNE1* and *CCNE2*, encoding cyclins E1 and E2. Whereas single knockout mice were viable, double knockout embryos died around midgestation. Strikingly, however, these embryos showed no overt defects in cell proliferation. Instead, we observed developmental phenotypes consistent with placental dysfunction. Mutant placentas had an overall normal structure, but the nuclei of trophoblast giant cells, which normally undergo endoreplication and reach elevated ploidies, showed a marked reduction in DNA content. We derived trophoblast stem cells from double knockout E3.5 blastocysts. These cells retained the ability to differentiate into giant cells *in vitro*, but were unable to undergo multiple rounds of DNA synthesis, demonstrating that the lack of endoreplication was a cell-autonomous defect. Thus, during embryonic development, the needs for E-type cyclins can be overcome in mitotic cycles but not in endoreplicating cells.
Keywords: cell cycle/cyclin E/endoreplication/placenta/trophoblast

Introduction

Cyclin-dependent kinases (CDKs) orchestrate progression through the cell cycle in all eukaryotes. Activation of CDKs depends upon association with their regulatory subunits, the cyclins, in specific pair-wise combinations. In vertebrates, two types of CDKs are active through the initial (G_1) phase of the cell cycle: the first includes CDK4 and CDK6, associated with D-type cyclins (e.g. Matsushime *et al*., 1992); the second is CDK2, associated with cyclin E (e.g. Dulic *et al*., 1992; Koff *et al*., 1992) and, upon S-phase entry, with cyclin A. Expression of D-type cyclins depends upon extracellular mitogens, whereas that of cyclins E and A displays an intrinsic cyclic behavior (for reviews, see Sherr and Roberts, 1999).

Both types of CDK complexes target the Retinoblastoma-family or 'pocket' proteins pRb, p107 and p130. These proteins negatively regulate cell cycle progression, mainly by binding to the E2F-family transcription factors. The function of cyclin D–CDK4/6 is dispensable in cells defective for either pRb alone (Guan *et al*., 1994; Lukas *et al*., 1995; Medema *et al*., 1995) or p107 and p130 together (Bruce *et al*., 2000). Similarly, binding of pRb proteins by the adenoviral oncoprotein E1A renders cells insensitive to the CDK4/6 inhibitor $p16^{INK4a}$ (Alevizopoulos *et al*., 1998). Thus, pocket proteins appear to be the essential substrates of cyclin D–CDK complexes. Cyclin E cooperates with D-type cyclins in achieving full phosphorylation and inactivation of pRb and p130, but not p107 (Beijersbergen *et al*., 1995; Xiao *et al*., 1996; Lundberg and Weinberg, 1998; Harbour *et al*., 1999). Unlike D-type cyclins, however, cyclin E appears to be required for S-phase entry in pRb-null cells (Ohtsubo *et al*., 1995) and can promote G_1–S progression independently from pRb and p130 phosphorylation (Alevizopoulos *et al*., 1997; Lukas *et al*., 1997; Kelly *et al*., 1998). In addition, in order to bypass cell cycle arrest by the CDK2 inhibitor $p27^{Kip1}$, E1A requires a second function besides pRb binding (Alevizopoulos *et al*., 1998). Collectively, these observations pointed to pRb-independent function(s) of cyclin E–CDK2 in G_1–S progression. Cyclin E–CDK2 phosphorylates two of its own regulators, CDC25A (Hoffmann *et al*., 1994) and $p27^{Kip1}$ (Sheaff *et al*., 1997; Vlach *et al*., 1997; see Discussion), suggesting positive feedback mechanisms. Other possible substrates include proteins involved in histone gene expression (NPAT: Ma *et al*., 2000; Zhao *et al*., 2000), centrosome duplication (nucleophosmin: Okuda *et al*., 2000), chromatin modifications (p300/CBP: Ait-Si-Ali *et al*., 1998; SWI/SNF: Shanahan *et al*., 1999), pre-mRNA splicing (Seghezzi *et al*., 1998) and DNA replication (for review, see Ewen, 2000). *In vitro* studies in mammalian and *Xenopus* cell-free systems have provided strong evidence for a direct role of cyclin E–CDK2 in regulating the initiation of DNA replication (Jackson *et al*., 1995; Krude *et al*., 1997; Furstenthal *et al*., 2001a,b; Coverley *et al*., 2002).

In contrast with cyclin E (hereafter referred to as E1), very few studies have directly addressed the function of cyclin E2. These two cyclins are closely related and display very similar cell cycle-regulated expression and biochemical properties, in particular activation of CDK2 and inhibition by p27 (Lauper *et al*., 1998; Zariwala *et al*., 1998; Gudas *et al*., 1999). Furthermore, the respective mRNAs are expressed in an overlapping manner during mouse embryonic development (Geng *et al*., 2001). To address the function of E-type cyclins, we knocked out their genes in the mouse. We report that these cyclins are redundant during development and have an essential

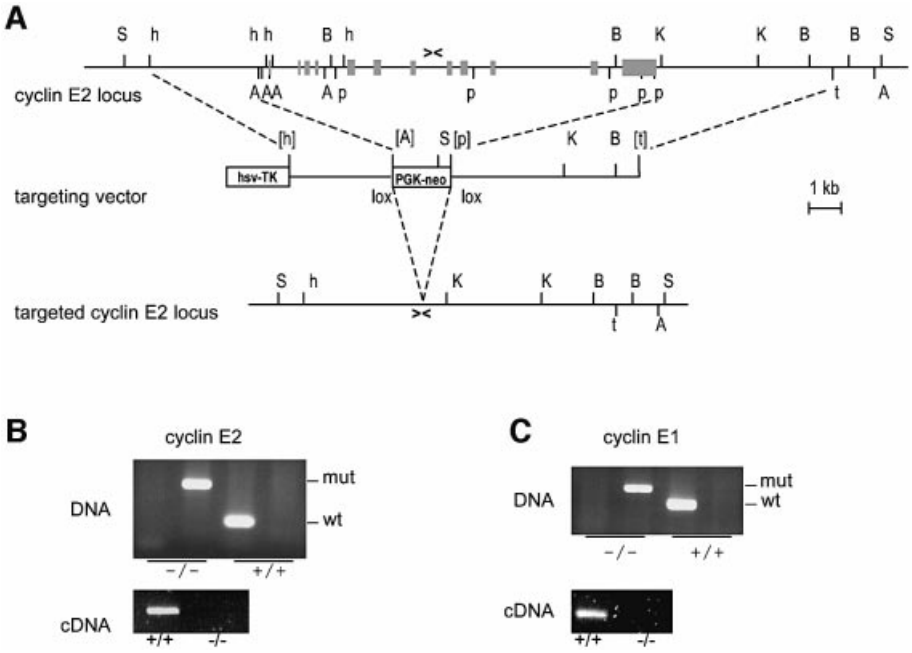

Fig. 1. Targeted disruption of *CCNE2 in* mice. (**A**) Schematic representation of the *CCNE2* locus (top), targeting vector (middle) and targeted allele (bottom). Gray boxes represent the exons. The arrowheads represent the PCR primers used to amplify the wild-type and targeted alleles for genotyping. Restriction enzymes: A, *Apa*I; t, *Aat*I; B, *Bam*HI; h, *Sph*I; K, *Kpn*I; p, *Spe*I; S, *Sac*I. The sites in brackets were used for cloning but lost in the process. See Materials and methods for experimental details. (**B** and **C**) PCR-based genotyping (top) and mRNA analysis (bottom) from *CCNE2* and *CCNE1* mutants, respectively. Genomic DNA was extracted from ear-punches and RNA from E13.5 mouse embryonic fibroblasts.

function in endoreplication of placental giant trophoblast cells, but not in embryonic cell cycles.

Results

Disrupting the cyclin E genes

To disrupt the murine cyclin E2 gene (*CCNE2*), genomic sequences spanning the 5′ UTR, the coding region and part of the 3′ UTR, were replaced by a *neo* cassette (Figure 1A). After electroporation of the targeting vector and G418 selection, correctly targeted cyclin E2$^{+/-}$ (129Sv) embryonic stem (ES) cells were identified by Southern blotting (data not shown). Selected clones were transiently transfected with a CRE expression vector to eliminate *neo* and subsequently injected in C56Bl/6 blastocysts, generating chimera that transmitted the mutated allele through the germline. The resulting cyclin E2$^{+/-}$ progeny in 129Sv/C56Bl/6 mixed background were then intercrossed, genotyped by PCR (Figure 1B, upper) and tested by reverse transcription and PCR for the absence of cyclin E2 mRNA (Figure 1B, lower). Viable cyclin E2-null mice were observed at a frequency (40/150) consistent with the expected Mendelian ratio (1/4) and exhibited no measurable growth impairment. Mutant females were fertile, whereas males exhibited partial sterility. Histological analysis of 2-month-old cyclin E2$^{-/-}$ males revealed that their testes were about two-thirds of the normal size, and the number of mature spermatozoa was reduced by 25–70%. In parallel with cyclin E2, we generated cyclin E1 mutant mice, starting with recombinant cyclin E1$^{+/-}$ ES cells bearing a *neo* cassette in place of the entire *CCNE1* coding region (a gift from Peter Sicinski; Geng *et al*., 2003). Genotyping and expression analysis of offspring (Figure 1C) revealed that viable cyclin E1-null mice were present at the expected frequency (52/216), developed normally and were fully fertile. To test whether cells lacking either cyclin E2 or E1 had measurable defects in proliferation, we isolated mouse embryonic fibroblasts (MEFs) from E13.5 embryos. MEF populations of either mutant genotype grew out as efficiently as wild-type control MEFs (data not shown) and retained a normal capacity to re-enter the cell cycle from quiescence (Figure 2). Altogether, the above results show that cyclins E1 and E2, taken individually, are dispensable for normal development and cell proliferation.

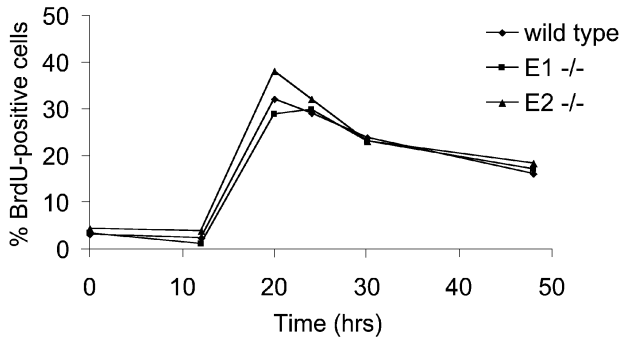

Fig. 2. Cyclin E1$^{-/-}$ and E2$^{-/-}$ single knockout cells are not impaired in cell cycle entry. The indicated wild-type and mutant mouse embryonic fibroblasts (from two independent preparations) were synchronized by serum starvation and contact inhibition, and subsequently released into cell cycle. DNA synthesis was monitored by flow cytometric analysis of DNA content and bromodeoxyuridine incorporation.

To determine whether E-type cyclins were redundant, we generated the cyclin E1$^{-/-}$E2$^{-/-}$ double knockout (dKO). Crosses of the single-mutant strains yielded E1$^{+/-}$

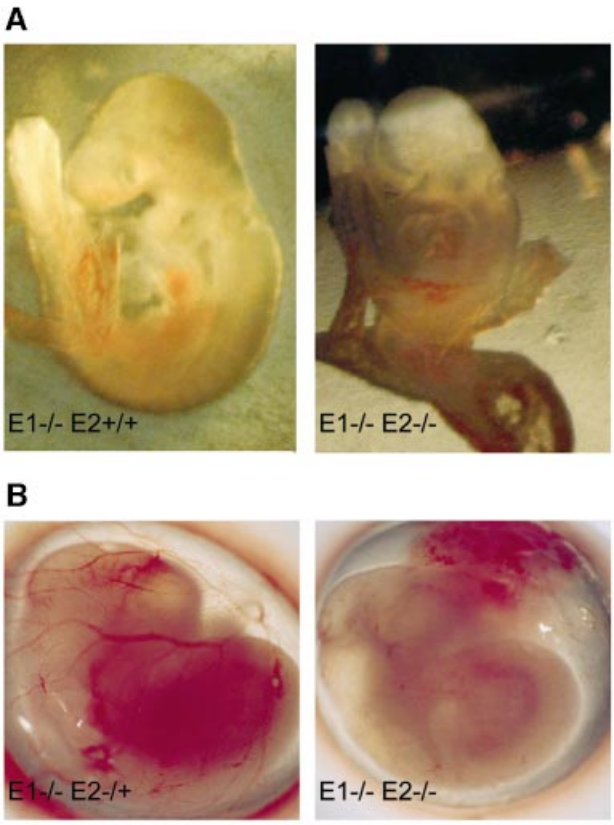

Fig. 3. Phenotypes of cyclin $E1^{-/-}E2^{-/-}$ double knockout (dKO) embryos. (**A**) E10.5 dKO embryos are smaller than control siblings. This relative difference in size was observed in different litters. (**B**) The vasculature in the dKO yolk sac at E11.5 is nearly absent. Note that the characteristic pattern of yolk sac vasculature in the control (left) is missing in the dKO (right).

$E2^{+/-}$ animals that were subsequently intercrossed. Of 189 newborns genotyped, none was a dKO (expected 1/16, or ⩾11 mice). All other genotypes were viable and observed at Mendelian ratios. In particular, $E1^{+/-}E2^{-/-}$ and $E1^{-/-}$ $E2^{+/-}$ animals developed like the respective single-mutant controls (hence, $E1^{+/-}E2^{-/-}$ males were partially sterile, like $E2^{-/-}$ males). To increase the frequency of the dKO genotype, subsequent breedings were derived from $E1^{-/-}$ $E2^{+/-}$ (of both sexes) and $E1^{+/-}E2^{-/-}$ animals (females only). Of >400 newborns genotyped, none was a dKO (expected 1/4, or ⩾100 mice). Thus, at least one E-type cyclin allele is required and sufficient for survival.

Mice lacking both cyclin E1 and E2 die from causes unrelated to cell proliferation

To determine the stage of lethality, we collected and genotyped embryos at various stages of development. At E11.5, dKO embryos were recovered at the expected Mendelian ratio (10/39) but most were dead, with only 2/10 showing a beating heart. At E10.5, dKO embryos were normally represented (16/73) and most of them were still alive. Thus, lethality occurred mainly between E10.5 and E11.5. The morphology of dKO embryos between E9.5 and E11.5 presented no obvious defects, but instead manifested a general developmental delay. dKO embryos were, with few exceptions, smaller and paler than their siblings (Figure 3A) and, in 60% of them, the yolk sac at E11.5 was poorly vascularized (Figure 3B). Furthermore, two E10.5 dKO embryos analyzed by whole mount staining with α-PECAM antibodies showed an arrangement of cranial vasculature typical of E9.5 embryos (data not shown). Altogether, these data suggest that dKO conceptuses (embryo and extra-embryonic tissues) suffer from general growth retardation and defects in vascularization.

To address whether cell proliferation was impaired in dKO embryos, we measured DNA synthesis *in situ* by bromodeoxyuridine (BrdU) staining of sections at E9.5–E11.5. Surprisingly, dKO embryos and their siblings showed no evident differences (Figure 4). Altogether, eight mutant embryos and matched controls were examined at different stages, with identical results (data not shown). This finding suggests that compensatory mechanisms operate in dKO cells to allow cell cycle progression (see Discussion). Understanding these mechanisms will require tractable cells, such as E13.5 MEFs. We have attempted to derive dKO cells at earlier stages, but have obtained very heterogeneous populations. Nonetheless, loss of E-type cyclins in dKO embryos causes no overt defects in cell proliferation at the time of lethality.

Requirement of cyclins E1 and E2 for endoreplication in trophoblast giant cells

There are various genes whose absence during development leads to extra-embryonic defects (e.g. Luo *et al*., 1997; Kraut *et al*., 1998), and for some of them the involvement in placental formation has been unexpected (Schorpp-Kistner *et al*., 1999; Tremblay *et al*., 2001). For instance, two recent papers describe extra-embryonic defects in mice lacking the cell cycle regulators DP1 and pRb (Kohn *et al*., 2003; Wu *et al*., 2003). Placental defects cause poor exchange of metabolites and oxygen, leading to secondary phenotypes such as developmental delay and yolk sac abnormalities, as seen in our dKO conceptuses. Histological analysis on sections of placentas stained with haemotoxylin and eosin (H&E) at stages E10.5 and E11.5 revealed a clear paucity in the nuclear DNA content of trophoblast giant cells (TGCs; Figure 5A), which was confirmed by quantitative Feulgen staining of DNA (Figure 5B). During differentiation of trophoblast stem cells (TSCs) into TGCs, multiple rounds of DNA synthesis occur without mitotic division (endoreplication) leading to increases in DNA content up to 1000N and to a parallel increase in nuclear size (Barlow and Sherman, 1972; Varmuza *et al*., 1988; Zybina and Zybina, 1996). TGCs are believed to be involved in the remodeling of the maternal uterus at implantation and, at later stages, for production of hormones and cytokines necessary for the maintenance of a proper embryo–mother interface (Cross *et al*., 1994; Cross, 2000). These key functions in placental development made TGCs prime candidates for the lethality of dKO embryos. Thus, we decided to address whether a decrease in endoreplication was a primary, cell-intrinsic defect of TGCs lacking E-type cyclins.

To achieve this goal, we derived TSCs from dKO and wild-type blastocysts (Tanaka *et al*., 1998). TSCs in the blastocyst constitute the polar trophoectoderm, maintained in an undifferentiated state by FGF4 produced by the underlying inner cell mass. TSCs are the precursors of the extra-embryonic ectoderm and the ectoplacental cone,

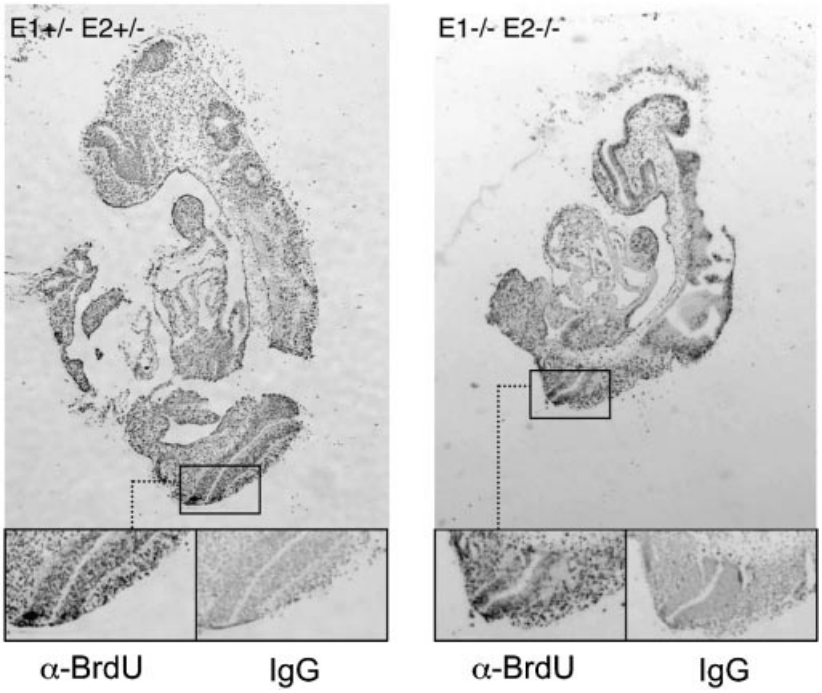

Fig. 4. Bromodeoxyuridine staining of E10.5 embryos shows equally abundant S phases in double knockout and control siblings.

which subsequently gives rise to the secondary giant cells, the labyrinth and spongiotrophoblast layers. When starved of FGF4, heparin and MEF-conditioned medium (MCM), TSCs give rise to all the trophoblast lineages *in vitro*, recapitulating *in vivo* placental processes (Tanaka *et al*., 1998). The trophoblasts committed to the TGC lineage adhere to the plate more tightly than the other cells and can be enriched by light trypsinization (Cross *et al*., 1995). Following this treatment, we initiated differentiation and allowed it to proceed for a total of 12 days, harvesting cells every 2 days. Analysis of DNA content, performed by flow cytometry (Figure 6A) or Feulgen staining (Figure 6B) gave striking results: unlike wild-type TSCs, dKO TSCs were unable to undergo multiple rounds of DNA replication. Only a few mutant cells reached a ploidy of 8N, whereas a large fraction of wild-type cells reached 8N and more (Figure 6A). dKO cells had not increased their DNA content after 26 days (data not shown), indicating they were not simply retarded in endoreplication and/or differentiation (see below).

Uncoupling TGC differentiation from endoreplication

Surprisingly, in spite of the differences in DNA content, the nuclei of wild-type and mutant TGCs reached similar sizes *in vitro* (Figure 6B) as well as *in vivo* (Figure 5), suggesting that these two parameters are not interdependent. The overall morphology of wild-type and dKO giant cells also appeared comparable (data not shown). These observations suggested that differentiation proceeded in mutant TSCs, in spite of the block in endoreplication. This concept was further supported by mRNA expression analysis, conducted by real-time PCR, for several differentiation markers characteristic of the different trophoblast cell lineages. Markers of TGC differentiation such as pl-1 (placental lactogen-1) and plf-1/proliferin (Lee *et al*., 1988; Faria *et al*., 1991) were similarly induced in wild-type and dKO cells (Figure 7A). Esx-1, a marker of the labyrinthine layer (Yan *et al*., 2000), showed elevated expression in dKO cells at early, but not later, time-points. Conversely, the spongiotrophoblast markers 4311 (tpbp) and flt-1 (Lescisin *et al*., 1988; Dumont *et al*., 1995) showed lower expression in dKO cells, suggesting that there may be defects in this lineage. Two markers of undifferentiated TSCs, eomes and cdx2 (Beck *et al*., 1995; Russ *et al*., 2000), were similarly downregulated in wild-type and mutant cells, confirming that differentiation was not blocked altogether.

Genes associated with progression through the mitotic cycle, such as CDC2 and cyclins A and B were downregulated upon differentiation in both wild-type and dKO cells (Figure 7B). CDK4 and CDK2 mRNAs were expressed at relatively constant levels in cells of both genotypes. CDK2 protein levels and CDK2-associated kinase activity were also retained in the mutant (data not shown), presumably due to association with cyclin A, which constitutes the bulk of CDK2 activity. The mRNAs for cyclins E1 and E2 showed a different pattern of regulation during TSC differentiation: E1 was downregulated (although still expressed at significant levels at day 12), whereas E2 mRNA levels remained constant.

Discussion

E-type cyclins are required for endoreplication in trophoblasts, but not for mitotic cycles in the mouse embryo

The deletion of a key cell cycle regulator would be expected to halt cell proliferation and block development at early stages, coincident with the depletion of maternal

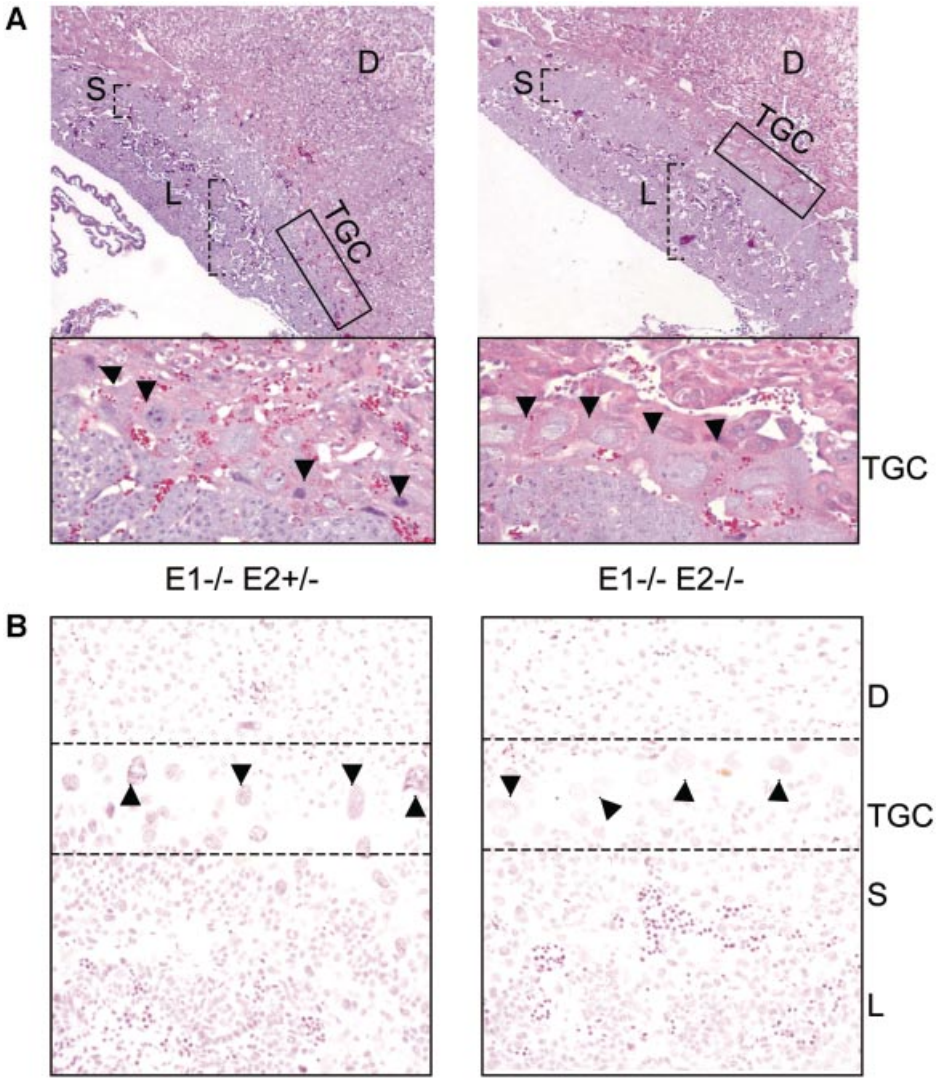

Fig. 5. Trophoblast giant cells in double knockout (dKO) placentas have a reduced amount of DNA. Haemotoxylin and eosin (**A**) and Feulgen (**B**) staining of sections through E10.5 placentas. The giant cell layer is either magnified in the inserts (A) or indicated by the dotted lines (B). The arrowheads show the giant nuclei: note the intense staining of nuclei in the control embryos, which is proportional to DNA content and is missing in the dKO. The placental layers are indicated as follows: D, decidua; TGC, trophoblast giant cells; S, spongiotrophoblast; L, labyrinth.

stores. For example, embryos lacking Max (the essential partner of Myc proteins) or cyclin A2 (the only A-type cyclin expressed in somatic cells) die shortly after implantation (E5.5–E6.5; Murphy *et al*., 1997; Shen-Li *et al*., 2000). As shown in this paper and in a parallel study (Geng *et al*., 2003), deletion of E-type cyclins yielded a dramatically different result. Cyclin $E1^{-/-}E2^{-/-}$ (dKO) embryos developed until midgestation (E10.5) and did not display any overt defect in cell proliferation. Furthermore, animals lacking the cyclin E and A partner CDK2 developed normally into adulthood (Ortega *et al*., 2003). Studies of cultured cell lines had already suggested that cyclin E–CDK2 activity could be dispensable for cell proliferation under certain conditions (Alevizopoulos *et al*., 1998; Tetsu and McCormick, 2003), but did not allow us to anticipate that normal development could occur without it. Although the new genetic data would suggest that cyclin E and CDK2 control no essential cell cycle transition, we deem it more likely that they do, but can be compensated for during development. We speculate that other components of the cell cycle machinery, such as cyclin A–CDK2, cyclin A–CDC2, cyclin D–CDK4, cyclin B–CDC2 or others are able to substitute in the different knockouts. In favor of such plasticity, mitotic cyclin B–CDC2 complexes have the potential to induce S-phase entry if targeted to the nucleus (Moore *et al*., 2003) and loss of two out of the three D-type cyclins can be compensated spatially and temporally in a developing mouse (Ciemerych *et al*., 2002).

Cyclin $E1^{-/-}E2^{-/-}$ (dKO) conceptuses showed developmental delays consistent with a placental dysfunction (see Results). At the histological level, the only defect that we detected in dKO placentas was a paucity of nuclear DNA staining in TGCs (trophoblast giant cells). By deriving TSCs (trophoblast stem cells) from dKO and wild-type blastocysts (E3.5), we demonstrated that the lack of endoreplication is an intrinsinc, cell-autonomous defect of dKO cells, whereas the TGC differentiation program *per se* is not blocked. In a parallel study, Geng *et al*. (2003) derived dKO ES cells and used the technique of tetraploid

complementation to obtain genetic evidence that the lethality of cyclin E1$^{-/-}$E2$^{-/-}$ embryos derives from a placental defect. Altogether, these data suggest that the endoreplicative defect of cyclin E1$^{-/-}$E2$^{-/-}$ TGCs is a direct cause of embryonic lethality.

In *Drosophila*, cyclin E functions in both endoreplicative and mitotic cycles (Knoblich *et al*., 1994; Duronio and O'Farrell, 1995; Sauer *et al*., 1995; Follette *et al*., 1998). It is therefore likely that a similar function is required during both types of cycles in vertebrates (see Introduction; MacAuley *et al*., 1998). One process that appears to be directly regulated by cyclin E in vertebrates is the initiation of DNA replication (Jackson *et al*., 1995; Krude *et al*., 1997; Furstenthal *et al*., 2001a,b; Coverley *et al*., 2002). In this regard, an important difference may exist between proliferating cells (i.e. that undergo successive mitotic cycles) and cells that re-enter the mitotic cycle after quiescence (or G_0). An elegant series of *in vitro* studies (Coverley *et al*., 2002) suggested that cyclin A alone may suffice to drive DNA synthesis in cycling cells, whereas S-phase entry from G_0 requires cyclin E in order to promote the formation of pre-replication complexes. Periodic (as opposed to continuous) expression of cyclin E is required for sustained endoreplication in *Drosophila* (Follette *et al*., 1998) and is also observed in rat TGCs (MacAuley *et al*., 1998). Furthermore, in both endoreplicative and mitotic cycles in *Drosophila*, cyclin E is required for the loading of mcm proteins onto replication origins (Su and O'Farrell, 1997, 1998). Thus, S-phase initiation in endoreduplicating cells may have requirements similar to those in cells that re-enter the cycle. Consistent with this hypothesis, E13.5 MEFs derived from rescued cyclin E1$^{-/-}$E2$^{-/-}$ mouse embryos displayed a selective defect in cell cycle re-entry (Geng *et al*., 2003). CDK2, instead, was dispensable for S-phase entry from G_0 and, by definition, for endoreplication, since mutant mice were viable (Ortega *et al*., 2003). In summary, mouse genetics indicate a requirement for cyclin E (as opposed to CDK2) in endoreplication and cell cycle re-entry, perhaps through a common biochemical mechanism. The fact that development of cyclin E1$^{-/-}$E2$^{-/-}$ embryos occurs until midgestation when they die due to placenta failure, and can even be rescued until birth (Geng *et al*., 2003), implies that cell cycle re-entry from quiescence is not critical during embryogenesis.

How do we reconcile mouse genetics with cell cycle textbooks?

Altogether, our data and the two parallel studies (Geng *et al*., 2003; Ortega *et al*., 2003) establish that cyclin E (i.e. E1 and E2) and CDK2 are dispensable for cell proliferation in the mouse. This contrasts with a large body of literature indicating that cyclin E–CDK2 is required for G_1–S progression (see Introduction). Another fundamental puzzle is raised by the difference in phenotypes between CDK2 and cyclin E1/E2 knockout mice, since only the latter die of placental defects. We speculate that a unique mechanism exists that will resolve these apparent discrepancies, and provide here a working hypothesis.

Mammalian cells that are growth-arrested almost invariably contain ternary complexes in which cyclin E–CDK2 is inhibited by the CKIs p21 or p27, whereas cyclin A is downregulated (e.g. Polyak *et al*., 1994; Sherr and Roberts, 1999). Ectopic expression of p21 or p27 reiterates this situation (e.g. Vlach *et al*., 1996, 1997; Sheaff *et al*., 1997). The CKIs possess distinct contact sites for cyclin E (or A) and CDK2, both of which are required for ternary complex formation and cell cycle arrest (e.g. Russo *et al*., 1996; Vlach *et al*., 1997). Thus, association of CKIs with cyclin E–CDK2 appears to be critical for cell cycle arrest.

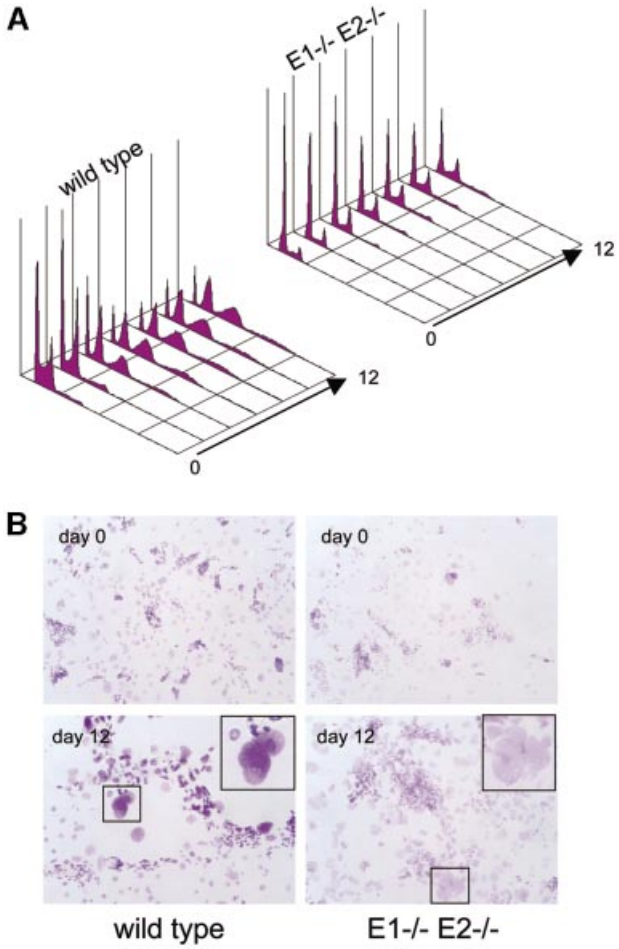

Fig. 6. Cyclins E1 and E2 are required for endoreplication in trophoblast giant cells (TGCs) *in vitro*. (**A**) Flow cytometric analysis of DNA content in nuclei of differentiating trophoblast stem cells (TSCs). (**B**) Feulgen staining of undifferentiated TSCs (day 0) and following differentiation into TGCs (day 12).

We propose that the dominant-inhibitory signal in cell cycle progression is the formation of the ternary cyclin E–CDK2–p27 (or p21) complex itself, rather than the mere loss of CDK2 catalytic activity. In this context, CDK2 kinase activity must be required to ensure that p27 is degraded in a timely manner. Indeed, one function of cyclin E–CDK2 is to phosphorylate p27, inducing its ubiquitination and degradation (Sheaff *et al*., 1997; Vlach *et al*., 1997; Montagnoli *et al*., 1999). Additional, phosphorylation-independent mechanisms exist to degrade p27 (Malek *et al*., 2001), but these may also be triggered by cyclin E–CDK2 (Furstenthal *et al*., 2001b). The critical difference between proliferating and quiescent (or endoreplicating) cells might be that the latter accumulate p27 (and hence ternary complexes) above a critical inhibitory

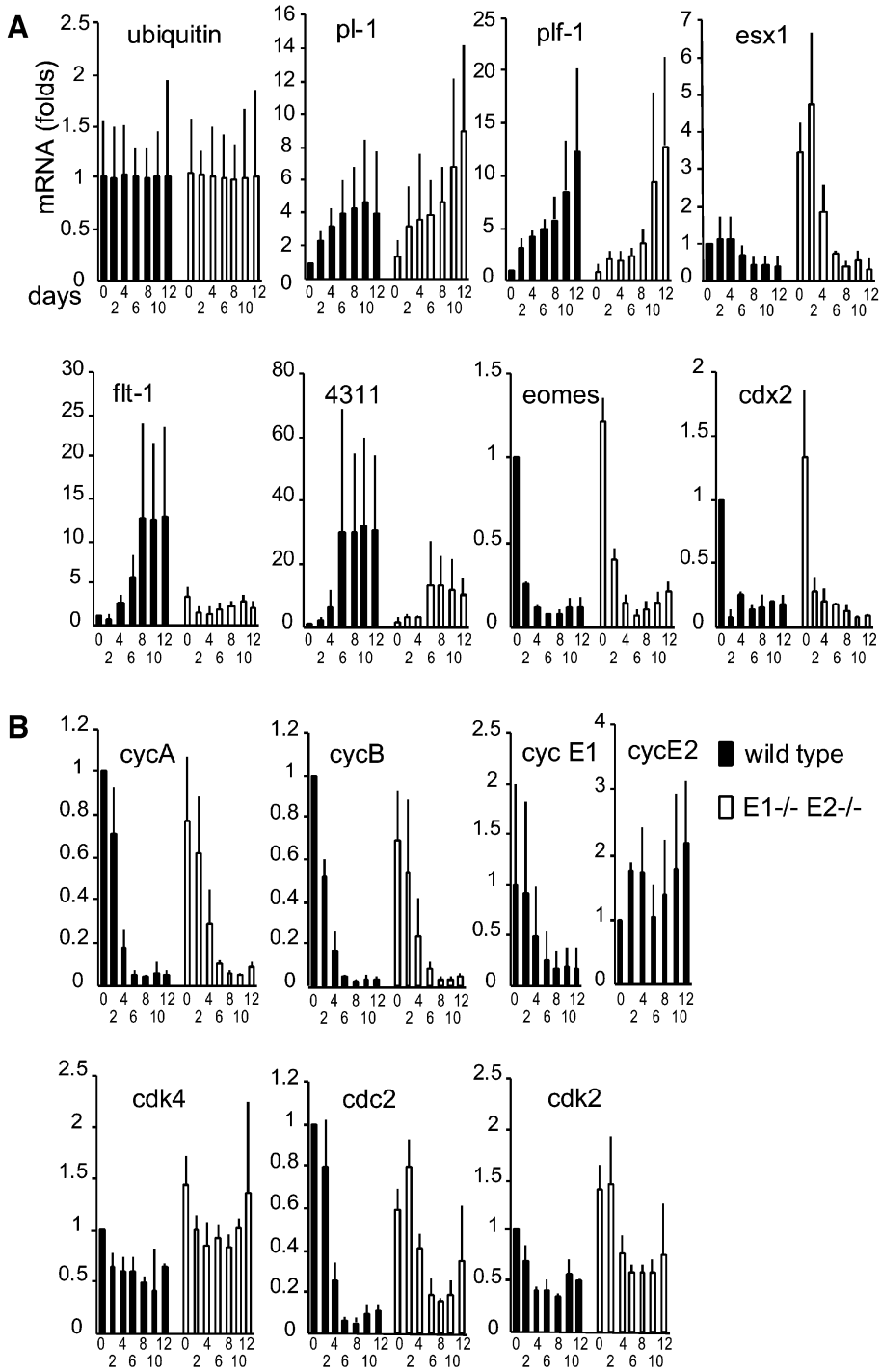

Fig. 7. mRNA expression analysis in differentiating trophoblast stem cells (TSCs). Wild-type and double knockout TSCs were induced to differentiate for 12 days. Every 2 days, RNA was isolated and expression of specific mRNAs was quantified with reverse transcription and real-time PCR. The results, normalized to ubiquitin, are expressed as fold-induction relative to day 0 in the wild-type, and represent the average of 3–5 different experiments from different TSC clones. (**A**) Ubiquitin mRNA (control) and placental markers. (**B**) Cell cycle genes.

threshold. In other words, continuously proliferating cells may not require cyclin E-induced degradation of p27.

This scenario would also account for the phenotypic differences between cyclin E1/E2- and CDK2-deleted mice. In cells lacking E-type cyclins, cyclin A–CDK2 is expected to be present. Cyclin A also forms inactive ternary complexes with CDK2 and p27 (e.g. Russo *et al*., 1996) but, unlike cyclin E, is unable to trigger p27 degradation (Sheaff *et al*., 1997; Vlach *et al*., 1997). In cells lacking CDK2, cyclin E appears to remain uncomplexed, but cyclin A is associated with kinase activity (Ortega *et al*., 2003). Hence, cyclin A–CDC2 (normally active at G_2–M) and/or other p27-resistant complexes might form in late G_1. Thus, the differential sensitivity of compensatory complexes to CKIs, including p27, p21 and the related $p57^{Kip2}$ in trophoblasts (Zhang *et al*., 1998),

Table I. Primers used for cDNA amplification

Ubiquitin (X51703)	TGGCTATTAATTATTCGGTCTGCAT and GCAAGTGGCTAGAGTGCAGAGTAA
pl-1 (M35662)	GCTGGTGTCAAGCCTACTCCTTT and GCAGTTGGTTTGGAGGACACAT
plf-1 (NM_031191)	ATAATCATCTCCAAAGCCACAGACA and CCGGACTGCGTTGATCTTTT
esx-1 (NM_007957)	TTGCAGAAGCACCGCAAG and AGCCGCCTCCAAGGTTTT
flt-1 (NM_010228)	GCTTTATAATAGCAAATGCAACGTACA and CGTTGACGGTGGCTTCG
4311 (NM_009411)	GAAGACTAACAAACTTCACAGTAGCGA and GAGCCTTCCGTCTCCTGGTC
eomes (XM_135209)	CCCATCAGATTGTCCCTGGA and CCGGGAAGAAGTTTTGAACG
cdx2 (NM_007673)	GCTCCGCAGAACTTTGTCAGT and GTAACCACCGTAGTCCGGGTAC
cyclin A (X75483)	AGTTTGATAGATGCTGACCC and TAGGTCTGGTGAAGGTCC
cyclin B1 (X64713)	ACTTCCTCCGTAGAGCATC and GCAGAGTTGGTGTCCATTC
cyclin E1 (X75888)	TGTTTTTGCAAGACCCAGATGA and GGCTGACTGCTATCCTCGCT
cyclin E2 (NM_009830)	GGAACCACAGATGAGGTC and CGTAAGCAAACTCTTGGAG
cdk4 (NM_009870)	GCTGGAGGCCTTTGAACATC and CCCGATCAGTTCGGGAAGTAG
cdk2 (NM_007673)	TCTGCTCTCACGGGCATTC and AGCTGGAACAGATAGCTCTTGATGA
cdc2 (U58633)	ACTCCAGGCTGTATCTCATC and CAAGTCTCTGTGAAGAACTCG

might explain why E-type cyclins are required, whereas CDK2 is dispensable for cell cycle re-entry and endoreplication. As a corollary, we predict that cells lacking CDK2, but not those lacking E-type cyclins, will escape p27- or p21-induced arrest. The key feature of this model is that it does not invoke CDK2-independent activities of cyclin E, although these remain a formal possibility.

Contrary to current models (Burdon *et al*., 2002), the knockout studies also imply that cyclin E and CDK2 are dispensable for proliferation of pluripotent stem cells. In particular, Geng *et al*. (2003) directly derived ES cells from cyclin $E1^{-/-}E2^{-/-}$ embryos. It remains to be addressed, however, whether cyclin E–CDK2 activity is rate-limiting for the exceptionally rapid cycling of ES cells, as suggested by a study with pharmacological inhibitors (Stead *et al*., 2002). The same study implied that the peculiar structure of the ES cell cycle, in particular the absence of a proper G_1 phase, depends upon regulators other than CDK2. We note that ES cells do not express p21 and p27 (Stead *et al*., 2002), consistent with our proposal that E-type cyclins are dispensable for cell cycle progression *per se* in the absence of these CKIs.

If cyclin E–CDK2–p27 complexes arrest the cycle, what are their targets? The sensitive downstream events may include transcription of E2F-target genes (such as cyclin A), the assembly of pre-replicative complexes (Furstenthal *et al*., 2001a,b) and/or any of the other processes in which cyclin E–CDK2 has been attributed a regulatory role (see Introduction). In certain tumor lines (Tetsu and McCormick, 2003) or upon expression of adenovirus E1A (Alevizopoulos *et al*., 1998), bypass mechanisms exist that render cells insensitive to inhibition of CDK2 by p27. Understanding these mechanisms is likely to shed new light on the functional targets of cyclin E–CDK2–p27.

Materials and methods

Generation of cyclin E1- and E2-deficient mice

A mouse genomic 129Sv λ GEM-11 library (see Radtke *et al*., 1999) was screened with a murine *CCNE2* cDNA probe. Genomic clones spanning 25 kb of the locus were obtained. To target *CCNE2*, a 3681 bp *Sph*I–*Apa*I fragment upstream of the first codon (5′ arm) was cloned in the TNLOX-1-3 vector (Radtke *et al*., 1999) between the hsv-thymidine kinase cassette and the PGK promoter-neo^r cassette. A 5511 bp *Spe*I–*Aat*II genomic fragment (3′ arm) was cloned downstream of PGK-*neo*^{r} (Figure 1A). Both arms are 99% identical to corresponding regions flanking *CCNE2 in* the annotated sequence of mouse (C57BL/6J) chromosome 4 (NT_039258). The endpoints are GCATGCCATACACTCGTTTA to CCTCCCGGAGGGGTGGGCCC (5′ arm) and ACTAGTTTGTCTGCCTTGCC to TACTCCAAGATTGTGACGTC (3′ arm). ES cells (129Sv) were electroporated with the linearized targeting construct and selected in G418 (400 μg/ml) and ganciclovir (2 μM). Proper recombination was confirmed by Southern blotting (data not shown) and resulted in deletion of a 12.3 kb genomic sequence spanning the 5′ UTR, all coding exons, as well as part of the 3′ UTR. Finally, transient transfection with a plasmid expressing CRE recombinase resulted in deletion of the *neo*^{r} cassette (Figure 1A). *CCNE1*-targeted ES cells, in which exons 2–11 were replaced with a *neo*^{r} cassette, were generated in the laboratory of Peter Sicinski (Geng *et al*., 2003) and kindly provided to us. Either *CCNE1*- or *CCNE1*-targeted ES cells were microinjected into C57BL/6 blastocysts. Male chimeras were obtained that transmitted either targeted allele into the germline. Genotypic analysis was performed by PCR on ear punches with the following primers: *CCNE1* wild-type allele: CGCCATGGTTATCCGGGAGATGG and CGCATACTGAGACACAGACT; *CCNE1* mutant: GATCTCTCGTGGGATCATTG and same as above; *CCNE2* wild-type: CAGTGCTCTTTGCAGCTGTATCA and GGATATTAATGTGTTCAACCCCTCA; *CCNE2* mutant: CGTCCTCTCCTGTCATTGGC and GAAATATGGCAAGGCAGACAAACTA.

Histology and BrdU incorporation

Conceptuses, still within the uterus or dissected at various stages of development, were fixed in 4% paraformaldehyde for 24 h and embedded in Paraplast Plus (Fisher Scientific). Paraffin sections (5 μm) were stained with H&E or Feulgen. For S-phase labeling, BrdU (100 μg/g body weight) was injected intra-peritoneally into pregnant females at 10.5 days post-coitum. The females were killed 2 h after injection. BrdU incorporation in embryos was detected on 5 μm paraffin sections with anti-BrdU antibodies (Becton Dickinson) used in combination with the Vectastatin ABC Kit (Vector Laboratories).

Isolation, culture and analysis of TSCs

Blastocysts were flushed out and collected from pregnant female uteri at E3.5 in M16 medium (Sigma Aldrich). Individual blastocysts were seeded onto mitomycin-treated MEFs, provided with 1 ng/ml FGF-4 and 1 U/ml heparin and cultured at 37°C with 5% CO_2. After initial expansion on MEF layers (4–5 passages), TSCs were cultured in 70% MCM and 30% fresh RPMI-1640 Glutamax (Gibco-BRL) supplemented with 1 ng/ml FGF-4 and 1 U/ml heparin (Tanaka *et al*., 1998). For preparation of MCM, E12.5 MEFs (isolated from CD-1 mice, Charles River Laboratory) were treated with 10 μg/ml mitomycin C for 3 h, trypsinized and replated at confluent density in RPMI-1640 Glutamax supplemented with 20% FBS (ES-cell screened and decomplemented), 50 μg/ml pen-strep, 1 mM sodium pyruvate and 100 μM β-mercaptoethanol. MCM was collected every 3 days for 9 days, filtered and frozen.

TSC differentiation was induced on the second day after passage. Cells were trypsinized for 2 min, and the remaining trypsin-resistant cells were cultured in differentiation medium (growth medium minus MCM, FGF-4 and heparin). For analysis of DNA content, differentiating TSCs (on 6 cm dishes) were harvested every 2 days by trypsinization, washed with PBS

and fixed in 70% EtOH. At the end of the differentiation time course (12 days), all collected samples cells were washed with PBS, fixed again with 1% formaldeyde, washed in PBS and treated with 0.08% pepsin in 0.1 N HCl for 20 min at 37°C. The resulting nuclei were washed once in 0.1 M Na-borate pH 8.0 and once in IFA (10 mM HEPES pH 7.4, 150 mM NaCl, 4% FCS, 0.1% sodium azide) supplemented with 0.5% Tween-20, resuspended in 1 ml of IFA containing 0.1 mg/ml RNase A and incubated at 37°C for 30 min. Propidium iodide was added (final 40 µg/ml), and nuclei were analyzed by flow cytometry.

mRNA expression analysis

RNA isolation from TSCs, cDNA preparation and real-time PCR were performed as described previously (Frank *et al*., 2001). The primers listed in Table I were used for cDNA amplification. For analysis of cyclin E1 and E2 mRNAs in MEFs, the same primers were also used in conventional PCR reactions (Figure 1B and C).

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