The Transcription Factor TCFAP2C/AP-2γ Cooperates with CDX2 To Maintain Trophoderm Formation

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In mammals, cell lineage specification is established at the blastocyst stage. At this stage, transcription factor Cdx2 represses pluripotency genes, thus promoting extraembryonic trophoblast fate. Recently, transcription factor Gata3 was shown to act in a parallel pathway in promoting trophoblast cell fate, suggesting that there are more factors working in the trophoblast lineage. Here, we report that the transcription factor Tcfap2c is expressed at a high level in the trophoderm and is able to induce trophoblast fate in embryonic stem cells. Trophoblast fate induced by Tcfap2c does not require Cdx2 and vice versa, suggesting that the molecules act in alternative pathways. However, both Tcfap2c and Cdx2 are required for the upregulation of Elf5, a marker of trophoderm stem cell maintenance, suggesting that both factors are required for stable trophoblast induction. Tcfap2c-induced trophoblast-like cells are stable in long-term culture, indicating that they are capable of self-renewal. Tcfap2c-controlled trophoblast maintenance involves the induction of Cdx2 and the repression of the pluripotency factor Nanog. Tcfap2c-induced trophoblast-like cells differentiate to trophoblast derivatives in vitro and contribute to the trophoderm in blastocysts in vivo. Taken together, these observations suggest that Tcfap2c and Cdx2 cooperate to override the pluripotency program and establish the extraembryonic trophoblast maintenance program in murine embryos.

The earliest cell fate decision during mammalian development is the establishment of the first two cell lineages of the blastocyst prior to implantation. The inner cell mass (ICM) forms the embryo proper as well as extraembryonic endodermal components of the placenta, whereas the trophectoderm (TE) gives rise to the fetal portion of the placenta, a structure unique to mammalian development. Self-renewing embryonic stem cell (ESC) and trophoblast stem cell (TSC) lines have been derived from each of these lineages. TSCs exhibit the potential to differentiate into multiple trophoblastic cell types in vitro, participate in the normal development of chimeras, and contribute exclusively to the trophoblastic component of the placenta in vivo. At the genetic level, key factors that establish and maintain the TE lineage in the early embryo have been identified. Based on so far unknown positional information, the Hippo signaling pathway component Yap (Yes kinase-associated protein 1, a coactivator of Tead4) is phosphorylated by Lats (large tumor suppressor, a Ser/Thr kinase that belongs to the Ndr/Lats subfamily of protein kinase A/PKG/PKC kinases) and becomes cytoplasmic in the inner cells of the morula. In outer blastomeres, Yap remains in the nucleus and associates with and activates TEAD4, which in turn transactivates the expression of the transcription factor Cdx2 (caudal-related homeobox 2). Cdx2 represses pluripotency markers such as Oct3/4 (Pou5f1) and Nanog and vice versa, which leads to the maintenance of the restricted expression of Cdx2 in the TE. CDX2 and the T-box factor Eomes (eomesodermin) are regarded as the key transcription factors required for the establishment of a functional TE. Recently, the transcription factor Gata3 was described to regulate trophoblast development downstream of Tead4 and in parallel with Cdx2. Restriction of lineage potency is mediated through the epigenetic regulation of Elf5, which is methylated and silenced in the embryonic lineage but hypomethylated and expressed in the trophoblast lineage. By forming a positive-feedback loop with Cdx2 and Eomes, Elf5 reinforces the commitment to the trophoblast lineage.

Little is known about the factors that control TSC maintenance and self-renewal or the molecules that control differentiation inhibition. Fibroblast growth factor 4 (FGF4) activation of ERK1/2 has been demonstrated to control TSC survival and self-renewal in a mitogen-activated protein kinase (MAPK) kinase kinase (MAP3K; MEKK)-dependent manner. In addition, members of the transforming growth factor-β/activin subfamily have been shown to influence TSC maintenance. The overexpression of Tead4, Cdx2, or Eomes in ESCs is sufficient to instructively promote trophoblast fate in ESCs and promotes the formation of functional TE cells from ESC cultures. There, the transcription factor Tcfap2c (AP-2γ;
Mouse Genome Informatics accession number 106032) was found to be upregulated in TSC-like cells (21). In mice and humans, the TCFAP2 (AP-2) family of transcription factors consists of five proteins, all of which have unique functions during mammalian development and act by controlling the balance between proliferation and differentiation (8). Tcfap2c-deficient mice die at approximately E7.5 due to trophodermal cell defects in the ectoplacental cone (2, 38). The mutant mice display reduced cell proliferation in the ectoplacental cone, and the number of trophoblast giant cells is reduced; therefore, the trophoblast fails to establish a functional placentating, leading to resorption of the embryo.

Here, we show that Tcfap2c is expressed in preimplantation embryos and becomes downregulated in the ICM of late blastocysts. Upon implantation, Tcfap2c is expressed in all TE derivatives, except syncytiotrophoblast cells of the labyrinth layer, by up to E19.5 of murine development. In vitro, TSC lines express TCFAP2C in an undifferentiated state as well as during differentiation into TE derivatives. Induction of Tcfap2c in ESCs (28) instructs the expression of TE markers and TSC fate even in Cdx2-deficient ESCs. Upregulation of Elf5 was demonstrated after either CDX2- or TCFAP2C-driven promotion of TSC fate in ESC. However, Elf5 expression is not observed in TSC-like cells generated from Cdx2-overexpressing Tcfap2c+/− ESCs or Tcfap2c-overexpressing Cdcd2+/− ESCs. Furthermore, we show that Tcfap2c-induced TSC-like cells retain their TSC character upon long-term culturing, demonstrating that Tcfap2c is sufficient to induce stable TSC fate. We demonstrate that these cells are functional based on their expression of markers of TE derivatives in vitro and the ability to incorporate into the TE of blastocysts in vivo. We provide evidence that TCFAP2C induces CdX2 and represses Nanog expression. This finding suggests that the maintenance of TSC fate requires both TCFAP2C and CDX2 protein function.

**Materials and Methods**

**Cell culture.** MG1.19 (12) ESCs were cultured as described previously (28). All other ESCs were grown on irradiated murine embryonic fibroblast (MEF) or feeders (1:100, clonal 88; Biocare Medical, Zytomed, Berlin, Germany), mouse anti-Oct3/4 monoclonal Ab (1:100; C-10; Santa Cruz, Heidelberg, Germany), rabbit anti-TCFAP2C polyclonal Ab (1:100; H77; Santa Cruz, Heidelberg, Germany), mouse anti-CDH3 monoclonal Ab (1:25; clone 56C1; Neomarkers, Fremont, CA), and rabbit anti-mouse NANOG (Abcam, Cambridge, United Kingdom). Detection of primary Abs was performed with secondary Abs that detect mouse and rabbit IgG, conjugated with Alexa Fluor 488 or Alexa Fluor 594 (1:1000; both from Invitrogen, Karlsruhe, Germany). 4′,6-Diamidino-2-phenylindole (DAPI; Sigma-Aldrich) was used for nuclear staining. Fluorescent images of the blastocysts were captured in optical sections using an Observer microscope (Carl Zeiss, Jena, Germany) with the apotome setting and were processed using Axiovision (version 4.7) software (Carl Zeiss). Cells were observed using an IM-DRB fluorescence microscope (Leica, Bensheim, Germany).

**Histology and immunohistochemistry.** Embryos were fixed in 4% neutral buffered formalin at 4°C overnight and embedded in paraffin. For immunohistochemistry, 1- to 3-μm sections were incubated using the following primary antibodies (Abs) in PBSTM: TCFAP2A (1:500; 1:200, clonal H9252; Biocare Medical, Zytomed, Berlin, Germany), mouse anti-Oct3/4 monoclonal Ab (1:100; C-10; Santa Cruz, Heidelberg, Germany), rabbit anti-TCFAP2C polyclonal Ab (1:100; H77; Santa Cruz, Heidelberg, Germany), mouse anti-CDH3 monoclonal Ab (1:25; clone 56C1; Neomarkers, Fremont, CA), and rabbit anti-mouse NANOG (Abcam, Cambridge, United Kingdom). Detection of primary Abs was performed with secondary Abs that detect mouse and rabbit IgG, conjugated with Alexa Fluor 488 or Alexa Fluor 594 (1:1000; both from Invitrogen, Karlsruhe, Germany). 4′,6-Diamidino-2-phenylindole (DAPI; Sigma-Aldrich) was used for nuclear staining. Fluorescent images of the blastocysts were captured in optical sections using an Observer microscope (Carl Zeiss, Jena, Germany) with the apotome setting and were processed using Axiovision (version 4.7) software (Carl Zeiss). Cells were observed using an IM-DRB fluorescence microscope (Leica, Bensheim, Germany).

**RT-PCR/quantitative real-time RT-PCR (qRT-PCR) analysis.** Total RNA was isolated from cells and tissues by using an RNeasy minikit (Qiagen, Hilden, Germany) or TRI reagent (Sigma-Aldrich), according to the manufacturer's instructions. For standard reverse transcription-PCR (RT-PCR), first-strand cDNA was synthesized from 500 ng or 1 μg of total RNA in a 20-μl reaction mixture using oligo(dt) primers and SuperScriptII reverse transcriptase (Invitrogen), according to the manufacturer's recommendation. PCR was performed using 25 to 30 cycles of 94°C for 30 s, 55 to 62°C for 30 s, and 72°C for 50 s. The primer sequences are available on request. Quantitative real-time PCR was performed using an ABI Prism 7300 sequence detection system (Applied Biosystems, Darmstadt, Germany) and SYBR green (Applied Biosystems), according to the manufacturer's protocol. Equivalent amounts of gDNA generated from the RT reactions were used as templates for PCR. Reactions were performed at least in triplicate for each sample. Expression of genes was normalized to the expression of b-actin and glyceraldehyde-3-phosphate dehydrogenase, and the mRNA level of each gene in untreated ESCs (control) was set equal to 1. The real-time PCR mixtures were processed in triplicate in a total volume of 20 μl containing 2 μl of cell-specific cDNA, 0.4 μl of Bst60R enzyme, and 10 μl of SYBR green master mix (Applied Biosystems). The PCR program was composed of 10 s at 95°C, followed by 45 cycles of 5 s at 95°C and 35 s at 60°C. After PCR, a melting curve analysis was arranged for determination of PCR product specificity. For each sample, a cycle threshold (Ct) value was recorded. PCRs were set up in triplicate, and the mean of the three Ct values was calculated. To determine the relative gene expression, the comparative Ct method (ΔΔCt method) was used. The level of gene expression was shown as the fold change compared to the level of the control sample. Primer sequences and references are available on request.

**Establishment of ESC carrying tamoxifen-inducible Tcfap2c.** The entire open reading frame of Tcfap2c was amplified by PCR with primers mAP2F1 (5′-GACGCCCAGTGTTGGAAAAAACA-3′) and mAP2R (5′-GGATCCCTTCGTCGTGTTTCCATTT-3′), followed by subcloning into the pCR2.1 vector (Invitrogen) and digestion with EcoRI. The Tcfap2c cDNA was subcloned into pCR2.1 lacking BamHI, followed by digestion with BamHI. The ligand binding domain of the mutant form of mouse estrogen receptor (ER) amplified by PCR with primers mERF (5′-GGATCCCGTTATTGAAATGGTTGCT-3′) and mESRR (5′-GGATCCTCAGATCGTGTTGGGGAAGCC-3′) was subcloned into the pCR2.1 vector, followed by digestion with BamHI and subcloning into BamHI of pCR2.1-Tcfap2c, resulting in the generation of pCR2.1-Tcfap2cER, followed by digestion with EcoRI. The cDNA was subcloned into EcoRI of pCAG-IP (28), resulting in pCAG-Tcfap2cER-IP. Thirty micrograms of pCAG-Tcfap2cER-IP was linearized by SacI and electroporated into 1 × 107 C17 ESCs at 575 V/cm and 200 μF using a Bio-Rad Gene-Pulser. Twenty-four hours after transfection, cells were selected for 4 days using 1.4 μg/ml puromycin. Clones in

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which pCAG-Tcfap2cER-IP had integrated into the genomic DNA were selected by their ability to undergo differentiation to TE by addition of 1 μg/ml of 4-hydroxy-tamoxifen (Tx; Sigma-Aldrich).

Establishment of ESCs carrying doxycycline-inducible Tcfap2c. A gene targeting kit obtained from Open Biosystems (Huntsville, AL) was used to generate single-copy transgenic ESCs by site-specific recombination using a method described previously (3). In short, pCR2.1-Tcfap2c containing the entire open reading frame of Tcfap2c was digested with EcoRI and the cDNA of Tcfap2c was inserted into pBS31_tetO_promoter/simian virus 40 t° of the tetO minimal cytomegalovirus (CMV) promoter. HK2 ESCs carrying the reverse tetracycline transcriptional activator (rtTA) transgene in the ROSA26 locus (3) were electrotransfected with 50 μg of pBS31_Tcfap2c and 25 μg of an expression vector for the rtTA recombinase (pCAGGS-rtTA). rtTA-mediated recombination of pBS31-Tcfap2c leads to the integration of Tcfap2c cDNA into the ColAI locus of HK2 ESCs. In parallel, this recombination initiates the expression of the promoter and ATG-les hygromycin resistance cassette present in this locus. Twenty-four hours after electrotransfection, 140 μg/ml hygromycin was added. Ten days later, colonies were picked and clones were screened by Southern blotting using SpeI to digest genomic DNA and a 3° internal probe as well provided with the kit. Two positive clones in which Tcfap2c expression is induced after addition of 0.5 μg/ml doxycycline for 48 h were expanded.

Production of chimeric embryos. Host embryos were obtained from C57BL/6 mice and were collected at the four-cell stage. Tcfap2cER-induced ESCs and Tcfap2cER-induced TSC-like cells were incubated for 10 min at 37°C in PBS supplemented with 1 μM fluorescent dye carboxyfluorescein succinimidyl ester (CFSE; Fisher Scientific, Schwerte, Germany). The staining procedure was stopped by adding 20% fetal bovine serum (Thermo Scientific HyClone, South Logan, UT). To generate chimeric embryos, 15 stained embryos were injected under the zona pellucida of four-cell-stage embryos as described previously (21). After injection, the embryos were first cultured in vitro in KSO medium lacking amino acids (15); after 24 h, they were transferred to DMEM supplemented with 2 mM glutamine, 50 μM penicillin and streptomycin, and 20 mmol/liter HEPES. They were visualized by fluorescence microscopy at the blastocyst stage using a Leica DM-IRB (Bensheim, Germany) inverted microscope. Image processing and merging were done using ImageJ freeware (version 1.37; National Institutes of Health [http://rsb.info.nih.gov/ij]). The embryos were examined and photographed using a Leica MZ stereomicroscope. Permission to perform animal experiments was granted from the Landesamt fuer Natur Umwelt und Verbraucherschutz, Recklinghausen, Germany (50.203.2-B12, 42/04).

Generation of ESCs deficient for Cdx2 or Tcfap2c. Heterozygous Cdx2 females were superovulated and mated with heterozygous males. The mouse line was a kind gift from Felix Beck (University of Leicester, Leicester, United Kingdom) (5). Blastocysts at 3.5 days postcoitum were flushed from the uteri in KSOM medium, treated with tyrode solution (to remove the zona pellucida), and plated onto mitomycin C-inactivated mouse embryonic fibroblast cells in standard ESC medium containing LIF. On day 4 to 5 after plating of the blastocysts, the blastocyst outgrowths that showed well-expanded ICMs with no apparent trophoblast cell compartment (a hallmark of Cdx2 deficiency) were picked and processed following a typical ESC derivation protocol (24). The newly established ESC lines were confirmed to be Cdx2−/− by PCR genotyping (5). For the generation of Tcfap2c-deficient ESCs, the heterozygous Tcfap2c animals generated by us (38) were used. The analogous protocol was followed to generate the Tcfap2c−/− ESC lines.

ChIP analysis. Wild-type TSCs (2 × 10⁶) were resuspended in 500 ml ice-cold PBS and cross-linked with 1% formaldehyde (Sigma-Aldrich) for 7 min at room temperature. The reaction was stopped by adding 0.1 ml 1.5 M glycerol, followed by washing the TSCs two times with PBS. The cell pellet was resuspended in 200 μl SDS lysis buffer (0.1% SDS, 10 mM EDTA, 50 mM Tris, pH 8.1), and the mixture was incubated for 10 min on ice. Sonication was performed in a Bioruptor apparatus (Diagenode, Belgium) for 10 min (high setting for 30 s on and 30 s off) to achieve a fragment size between 200 and 500 bp. After pelleting of the cells, the supernatant was transferred to a new tube and the DNA concentration was measured. Ten microliters of protein G Dynabeads (Invitrogen) was preincubated with 1 μg of specific anti-TCFAP2C antibody (H77; Santa Cruz) in 500 μl chromatin immunoprecipitation (ChIP) dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl, 167 mM NaCl, pH 8.1) for 1 h. After the supernatant was removed, the beads were washed two times in ChIP dilution buffer. For the precipitation, 10 μg of shared DNA, 2 μg salmon sperm DNA, and protease inhibitor were mixed in a total volume of 200 μl in ChIP dilution buffer and the mixture was incubated overnight at 4°C. On the next day, the protein G-antibody-DNA complex was washed three times in 0.5 ml wash buffer (0.01% Tween 20 in Tris-EDTA [TE]). The DNA was eluted by adding 110 μl elution buffer to the pelleted complex at room temperature for at least 1 h. Reverse cross-linking was performed by adding 5 μl proteinase K and 10 μl 5 M NaCl and incubation at 55°C for 2 h, 65°C for 6 h, and 75°C for 2 h. Ethanol-sodium acetate-precipitated DNA was analyzed by PCR using the primer pair F (5′-CAGTGGGTCACCTAAGCCT-3′) and R (5′-GACACACAAAATTCGATCT-3′). As a control, ChIP was performed without antibody and an antihemagglutinin (anti-HA) antibody.

 Luciferase assay. Regulatory reporter elements of the respective genes were amplified by PCR from 129sv genomic DNA (the primers are available on request), subcloned into the pCIIE TOPO vector (Invitrogen), and verified by sequencing. The luciferase reporter plasmids were constructed by the introduction of the regulatory elements into pG3-Lucis (Promega, Madison, WI). For generating the dTFCAP2F-luciferase reporter, the Nanog promoter was digested with EcoRI and Xhol and religated to delete the sequence between bp 310 and 118. A total of 75 ng of luciferase reporters and 125 ng of the expression vectors encoding Tcfap2c (pCAG-Tcfap2c-IP) and Cdx2 (pCAG-Cdx2-IP; a gift from Hitoshi Niwa, RIKEN Center for Developmental Biology [CDB], Kobe, Japan) were transfected into 2.5 × 10⁴ MGL1.19 ESCs using Lipofectamine 2000 (Invitrogen) with 4 μg of pRL-CMV (Promega) as an internal control. The transfected cells were lysed 24 h after transfection, and luciferase activity was estimated using a dual-luciferase assay kit (Promega). The activity of the luciferase reporter co-transfected with the empty pCAGIP expression vector was set equal to 1.

RESULTS

Tcfap2c expression was detected in early embryos in the trophoblast lineage. To determine the spatiotemporal expression of Tcfap2c and to gain better insight into the role of Tcfap2c in early development, we analyzed the mRNA and protein levels at various preimplantation stages. Using RT-PCR, we detected Tcfap2c mRNA in morulae and blastocysts (Fig. 1A), confirming the results presented by Winger et al. (39). Immunodetection showed that the TCFAP2C protein is present in all cells of the eight-cell stage and morula (data not shown). By the late blastocyst stage, the TCFAP2C signal was high in the trophoderm and reduced in the inner cell mass (Fig. 1B, B′, and B″). After implantation, on days E5.5 and E6.5, the TCFAP2C protein was detected in all cells of the TEC lineage, such as the extraembryonic ectoderm, which harbors the trophoblast stem cell population. TCFAP2C was also found in the eocplacental cone and in primary and secondary trophoblast giant cells but not in the embryo proper or primitive endoderm (Fig. 1C and D). On day E7.5, TCFAP2C persisted in all cells of the TEC lineage. The thin layer of mesothelial cells underlying the chorion and the cells of the primitive endoderm were negative for TCFAP2C (Fig. 1E and E′). On day E8.5, after chorioallantoic fusion, the TCFAP2C protein was not detected in the allantois compartments, which give rise to placental blood vessels and the umbilical cord (Fig. 1F and F′). Beginning at E11.5 and continuing to E19.5, the TCFAP2C protein was detected in the developing spongiotrophoblast and giant cell layers (Fig. 1, G) but not in the syncytiotrophoblast cells of the labyrinth layer. On E19.5, giant cells and spongiotrophoblast cells express TCFAP2C, while in the labyrinthine layer, only a few small mononuclear cells were positive for TCFAP2C. Immunodetection of adjacent sections using isocitrate B4, which detects maternal blood sinusoids, shows that TCFAP2C-positive cells remain in contact with isocitrate B4-positive cells and are therefore most likely mononuclear giant cells. This finding is further supported by the fact that mononuclear giant cells are known to express placental lactogen II (PL2), which has been demonstrated to be a transcriptional target of TCFAP2C in rat and human cells (29, 32). Next, immunostaining of ESCs and TSCs was performed.
Interestingly, few cells within ESC colonies were positive for TCFAP2C (Fig. 2A and B). In contrast, TSCs displayed a strong TCFAP2C signal (Fig. 2C and D). Western blot analyses for the detection of all TCFAP2 isoforms demonstrated that TCFAP2C and TCFAP2A were expressed at high levels in undifferentiated TSCs. Upon induction of TSC differentiation, the TCFAP2C and TCFAP2A signals were upregulated and persisted up to day 13 of differentiation (the last day of analysis). Also, by this time, terminally differentiated spongiotrophoblast, syncytiotrophoblast, and giant cells were detected in culture (data not shown). In addition, the TCFAP2B protein was detected as soon as differentiation was initiated. In contrast, the TCFAP2D and TCFAP2E proteins were not detected in TSCs (Fig. 2E). The specificities of the TCFAP2A and TCFAP2C antibodies were confirmed using in vitro-translated protein (data not shown). These data suggest a role for TCFAP2A, TCFAP2B, and TCFAP2C in TSCs.

Activation of Tcfap2c is indispensable for TSC establishment and maintenance. Tcfap2c-deficient mice display a defect in trophoderm development, as determined by gene-knockout studies (2, 38); therefore, we tried to generate TSCs from Tcfap2c-deficient embryos for detailed analyses. Here, 22 blastocysts from Tcfap2c+/− intercrosses were cultured under TSC proliferation-permissive conditions (36). All isolated blastocysts attached to the dish and initiated characteristic trophodermal outgrowths. The outgrowths from Tcfap2c+/+ and Tcfap2c+/− blastocysts were indistinguishable, whereas the outgrowths from Tcfap2c−/− embryos showed an altered morphology, consistent with the findings of previous studies (2, 38) (data not shown). After passage 5, genotyping indicated that we obtained 13 Tcfap2c+/+, 5 Tcfap2c+/−, and 4 Tcfap2c−/− TSC lines. However, the cell lines heterozygous or homozygous for the Tcfap2c deletion could not be maintained. These mutant cells ceased to proliferate, and large, flattened cells appeared, which is indicative of differentiation (data not shown). Consequently, after 10 passages, genotyping revealed that only TSC lines wild type for Tcfap2c alleles were established (n = 13). Because Tcfap2c−/− ESCs were derived from blastocysts (described later), these results suggested that Tcfap2c is not essential for initial outgrowth. Instead, Tcfap2c may be required for the maintenance of TSCs but is dispensable for the derivation of ESC cultures. Moreover, even a reduction in the gene dosage to one intact allele compromised TS maintenance.

Expression of Tcfap2c in ESCs induces expression of TEC markers. It has been shown that the expression of TEC marker
for mesenchyme differentiation. Brachyury, was reduced and the primitive endoderm markers, Gata6 and Hnf4b, were not detected, indicating that Tcfap2c expression is sufficient to promote trophoblast fate in ESCs (Fig. 3I, green bars). Of note, during differentiation, few colonies displaying the ESC morphology remained; however, they were still positive for NANOG, while the surrounding cells expressed the TSC marker CDX2 (data not shown). Cells expressing both CDX2 and NANOG or CDX2 and OCT3/4 were not observed (data not shown). In addition, two ESC lines harboring doxycycline-inducible Tcfap2c were established. Most experiments performed with Tx-inducible Tcfap2c lines were repeated with the doxycycline-inducible lines (data not shown) to exclude the side effects from tamoxifen or doxycycline and showed identical results.

The caudal related homeoprotein CDX2 has been reported to play a central role in TEC specification in vivo and in TSC instruction in ESC-based systems (28, 37). To examine the epistatic relationship between Cdx2 and Tcfap2c, we established Cdx2−/− ESCs that conditionally express Tcfap2cER (Cdx2−/− Tcfap2cER). After treatment with Tx under TSC conditions, multiple independent clones exhibited trophoblast-like morphologies (Fig. 3G) and expressed CDH3 (Fig. 3H and I). Quantitative real-time PCR experiments showed that the pluripotency markers Oct3/4 and Nanog were reduced and that the markers Eomes, Bmp4, Gata3, Tcfap2a, Hand1, Pl1, and Pl2 were induced to levels lower than those in Cdx2 wild-type cells (Fig. 3J, compare the red and purple bars). These results indicated that Tcfap2c acts independently of Cdx2 to induce TE fate.

Next, a Tx-inducible Cdx2 transgene (Cdx2ER, obtained from H. Niwa) was introduced into wild-type and Tcfap2cER−/− ESCs (28). Again, after treatment with Tx, the cultures exhibited trophoblast-like morphologies and expressed TSC markers (data not shown). qRT-PCR was performed to analyze the levels of Cdx2 and Tcfap2c in the Tx-inducible cell lines used in the experiments. Of note, neither the Tcfap2c nor the Cdx2 levels achieved by the Tcfap2cER or Cdx2ER transgene reached levels comparable to the endogenous Tcfap2c and Cdx2 levels in TSCs (data not shown). Taken together, these experiments indicated that Tcfap2c can be used to promote the generation of TSC-like cells from ESCs in a Cdx2-independent manner, and vice versa.

After the embryonic and trophoblast cell lineages were specified, a stable maintenance of lineage identity is ensured by epigenetically imposed cellular memory (33). This lineage restriction is primarily mediated by the transcription factor Elf5. Elf5 is hypomethylated and expressed in the TE in late blastocysts, is methylated, and is repressed in the ICM (25). Elf5 maintains expression of the trophoblast stem cell gene Cdx2 (and Eomes), reinforcing trophoblast cell fate. During TSC differentiation, the Elf5 signal is very rapidly lost (25).

Using ESCs harboring the Tcfap2cER- and Cdx2ER-inducible alleles, we asked if Elf5 expression is activated during the promotion of the TSC-like morphology. Both transgenes were able to induce Elf5 in ESCs that were wild type for the Cdx2 and the Tcfap2c loci. However, the Elf5 signal was very weak in Cdx2−/− Tcfap2cER or Tcfap2cER−/− Cdx2ER cells (Fig. 4A), and this result was confirmed by qRT-PCR analyses (Fig. 4B). Induction of Elf5 by Tcfap2c or Cdx2 overexpression was ac-

### Figure 2: Expression of TCFAP2C in cell culture.

Five panels (A to D) Photomicrographs of ESC colonies (A and B) and TSC colonies (C and D). (A and C) Immunohistochemical detection of TCFAP2C (green). (B and D) Bright-field photograph. The red circles in panels A and B indicate the locations of the ESC colonies. (E) Western blot detecting all TCFAP2 proteins in TSCs grown in CMF4H and following differentiation after the removal of heparin and FGF4 (2 to 13 days). The blot shows the locations of the ESC colonies. (E) Western blot detecting all TCFAP2 proteins in TSCs grown in CMF4H and following differentiation after the removal of heparin and FGF4 (2 to 13 days). The blot shows the locations of the ESC colonies.

### Figure 3: Generation of TSC-like cells from ESCs.

We generated ESCs stably expressing a Tx-inducible fusion between Tcfap2c and a modified ligand binding domain of the estrogen receptor (Tcfap2cER). These cells remained ESCs when they were grown under ESC conditions without Tx (Fig. 3A) and underwent random differentiation under TSC conditions without Tx (Fig. 3B). Using trophoblast stem cell culture conditions (CMF4H) (36) and Tx, TSC-like cultures were derived from ESCs (28). Again, after treatment with Tx, the cultures exhibited trophoblast-like morphologies and expressed TSC markers (data not shown). qRT-PCR was performed to analyze the levels of Cdx2 and Tcfap2c in the Tx-inducible cell lines used in the experiments. Of note, neither the Tcfap2c nor the Cdx2 levels achieved by the Tcfap2cER or Cdx2ER transgene reached levels comparable to the endogenous Tcfap2c and Cdx2 levels in TSCs (data not shown). Taken together, these experiments indicated that Tcfap2c can be used to promote the generation of TSC-like cells from ESCs in a Cdx2-independent manner, and vice versa.

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### Figure 3: Expression of TCFAP2C in cell culture.

(A to D) Photomicrographs of ESC colonies (A and B) and TSC colonies (C and D). (A and C) Immunohistochemical detection of TCFAP2C (green). (B and D) Bright-field photograph. The red circles in panels A and B indicate the locations of the ESC colonies. (E) Western blot detecting all TCFAP2 proteins in TSCs grown in CMF4H and following differentiation after the removal of heparin and FGF4 (2 to 13 days). The blot shows the locations of the ESC colonies. (E) Western blot detecting all TCFAP2 proteins in TSCs grown in CMF4H and following differentiation after the removal of heparin and FGF4 (2 to 13 days). The blot shows the locations of the ESC colonies.
accompanied by trophoblast differentiation, as shown by the appearance of giant cells and the upregulation of Hand1 (Fig. 4B). The lack of the early TSC marker Eomes (Fig. 3J) and the low level of Elf5, along with morphological evidence, indicate that the TSC-like cells lacking Cdx2 or Tcfap2c are not able to maintain the undifferentiated TSC-like state (Fig. 3H). In agreement with this notion, all previous attempts to derive permanent TSCs from Cdx2−/− or Tcfap2c−/− blastocysts repeatedly failed (35; data not shown). These results suggested that the maintenance of undifferentiated TSC-like cells, as measured by Elf5 expression, requires both Tcfap2c and Cdx2. Next, we analyzed if Tcfap2cER-induced TSC-like cells are stable in culture. To this end, we treated Tcfap2cER-induced ESCs for the first 10 days with Tx to initiate TSC-like cultures.

FIG. 3. Tcfap2c induces TSC fate in a Cdx2-independent manner. (A) Colony morphology of Tcfap2c-induced ESCs cultured in ESC medium. (B) Tcfap2cER-induced ESCs cultured for 7 days in CM4H without Tx. (C) Formation of giant cells from Tcfap2cER-ESC after the addition of Tx in TSC medium without FGF4 and heparin. (D to F) In vitro culture of Tcfap2cER-induced ESCs under CMF4H and Tx conditions for 7 days. (F) Expression of the TS marker CDH3 (placental cadherin) was detected with anti-CDH3 antibody (green), and the nuclei were stained with DAPI (blue). (G to I) Culture of Cdx2+/− Tcfap2cER-induced ESCs under CMF4H and Tx conditions for 7 days. (A to I) Scale bars, 50 μm. (J) Quantitative RT-PCR analysis of indicated marker gene expression in ESCs (blue), TSCs (red), Tcfap2cER-induced ESCs (green), and Cdx2+/− Tcfap2cER-induced ESCs (purple). Total RNA was prepared from each pool at day 7 after activation with Tx. The markers analyzed are indicated.
Here, multiple colonies with a TSC-like morphology were de-
rived. Thereafter, the cells were kept in CMF4H medium without Tx. The colony at passage 5 is shown as a bright field (C). Expression of the TSC marker cadherin3 (pCadherin) was detected with the anti-CDH3 antibody (green), and the nuclei were stained with DAPI (blue) (D).

FIG. 4. Maintenance of TSC fate requires Tcfap2c and Cdx2. (A) RT-PCR analysis detecting Elf5 in TSC-like cells with the indicated genotype produced by adding Tx for 7 days compared to the Elf5 signal in ESCs and TSCs. (B) Quantitative real-time PCR of the same cells detecting Elf5 and Hand1. Photomicrograph of TSC-like colonies which had been induced by Tx in Tcfap2cER-induced ESCs for 7 days in CMF4H. Afterwards, cells were grown in CMF4H without Tx. The colony at passage 5 is shown as a bright field (C). Expression of the TSC marker cadherin3 (pCadherin) was detected with the anti-CDH3 antibody (green), and the nuclei were stained with DAPI (blue) (D).

Thereafter, the cells were kept in CMF4H medium without Tx. Here, multiple colonies with a TSC-like morphology were derived and maintained for more than 10 passages. Interestingly, the compact TSC-like cultures generated by Tcfap2c induction were always surrounded by large, flat, multinucleated cells (Fig. 4C and D), which indicates ongoing giant cell differentiation.

Tcfap2c-induced TSCs have the ability to differentiate along the trophectodermal lineage in vitro and in vivo. Quantitative real-time PCR analyses showed that the TSC-like cells produced by Tcfap2cER induction expressed markers of undifferentiated extraembryonic ectoderm (ExE) (Mash2), trophoblast giant cells (Pl1), and spongiotrophoblast cells (Tbpba). However, the level of Mash2 in Tcfap2cER-induced TSC-like cells was much lower than that in Cdx2ER-induced TSC-like cells and the levels of markers of differentiated TE (Pl1 and Tbpba) were much higher (Fig. 5A). This finding is consistent with the morphological data (Fig. 4C and D), since the Tcfap2cER-induced TSC-like cells tend to differentiate more easily than Cdx2ER-induced cultures. We demonstrated earlier that Tcfap2c is expressed in all cells of the extraembryonic lineage except syncytiotrophoblast cells. If the Tcfap2c-induced TSC-like cells resemble true TSCs, one would expect the cells to also differentiate into syncytiotrophoblast cells. Alternatively, Tcfap2c induction might generate precommitted TSC-like cells, which would be unable to differentiate into syncytiotrophoblast cells. TSC-like cells were first generated by Tx-mediated Tcfap2c induction. Thereafter, Tx was omitted, and the TSC-like cultures were treated with TSA to promote syncytialization in vitro (22). The Tcfap2c-induced TSC-like cells expressed the syncytiotrophoblast marker Syncytin A (Fig. 5B), indicating that their differentiation potential is not restricted and that they can be considered true TSCs.

We next addressed if TSC-like cells generated by the expression of Tcfap2c in ESCs are functional in vivo. We labeled TSC-like cells induced by Tcfap2cER with the CFSE fluorescent dye and injected them into four-cell-stage embryos to follow their fate in blastocysts. Of a total number of 18 injected embryos, 8 developed to the blastocyst stage. All cells contributed to the TE but not the ICM (Fig. 5C). Injection of control Tcfap2cER-induced ESCs that had been cultured in ESC medium without Tx contributed to the ICM (of 10 ESCs injected, 6 survived, 5 showed ICM staining, and 1 showed no staining) (Fig. 5D). This result suggests that Tcfap2c-induced TSC-like cells resemble TE cells in their ability to integrate into the trophectoderm of blastocysts.

TCFAP2C and CDX2 regulate each other, and TCFAP2C represses Nanog. Transfac and GenBank analyses were used to determine the locations of TCFAP2C binding sites in the promoter elements of Cdx2, Tcfap2c, Eomes, Fgfr1c, Nanog, and Oct3/4. Next, we evaluated the TCFAP2C transactivation of promoter elements of Cdx2, Tcfap2c, Eomes, and placenta-specific Fgfr1c and compared the results to those
and H11011icated chromatin containing Tcfap2c recruitment of oligonucleotides to amplify a fragment of the (Fig. 6C, arrowheads P1 and P2). This method allowed us to, were subjected to PCR using the depicted primers Tcfap2c which had been subjected to IP using antibodies specific for manner on the ATG) resulted in a moderate loss of repression, indicating little effect on Eomes and the Fgfr2c promoter (Fig. 6A, black bars); however, the levels of activation were lower than those from CDX2-mediated transactivation (Fig. 6A, white bars). When we analyzed the effect of TCFAP2C on the promoters of pluripotency markers, we found that the Nanog reporter construct was repressed (the value for the control was set equal to 1) by TCFAP2C and CDX2. In contrast, Oct3/4 displayed only mild repression by TCFAP2C (Fig. 6B, black bars). Deletion of the three putative binding sites for TCFAP2 at positions −313, −294, and −202 in the Nanog promoter (relative to the position of ATG) resulted in a moderate loss of repression, indicating that TCFAP2C may act in a direct manner and an indirect manner on the Nanog promoter. Next, we assessed the recruitment of Tcfap2c to these sites via ChIP analyses. Sonicated chromatin containing ~300-bp fragments of DNA, which had been subjected to IP using antibodies specific for Tcfap2c, were subjected to PCR using the depicted primers (Fig. 6C, arrowheads P1 and P2). This method allowed us to amplify a fragment of the Nanog promoter between nucleotides −397 and −167 that contains the three putative TCFAP2 binding sites. ChIP with an anti-TCFAP2C antibody showed protein-DNA complex formation with the Nanog promoter element, indicating that TCFAP2C bound to the sites, suggesting direct transcriptional regulation (Fig. 6D, lane Tcfap2c). Thus, TCFAP2C regulates the trophectoderm markers Cdx2 and Tcfap2c and represses the pluripotency marker Nanog.

**DISCUSSION**

Our results demonstrate a novel role for Tcfap2c in the establishment and maintenance of the TE. Tcfap2c is expressed in the TE of late blastocysts and in all cells of the TE lineage except syncytiotrophoblast cells. Furthermore, Tcfap2c and Tcfap2a are expressed in TSC cultures. Tcfap2c-deficient blastocysts did not give rise to self-renewing TSCs but were capable of ESC formation, indicating an essential role for Tcfap2c in the maintenance of TSC fate in vitro. Tcfap2c is sufficient for the induction of TSC-like fate when it is expressed in ESCs in FGF4, heparin, and fibroblast-conditioned medium, even in the absence of Cdx2. We showed that Tcfap2c-induced TSC-like cells are functional TE cells based on their expression of markers of TE derivatives in vitro and the ability to integrate into the trophectoderm layer of blastocysts in vivo. The induction of Tcfap2c in ESCs for 10 days was sufficient to generate stable and self-renewing TSC-like cell lines that were maintained for more than 10 passages under regular TSC conditions. However, loss of either Tcfap2c or Cdx2 leads to the failure of Elf5 to be upregulated in TSC-like cells and their subsequent differentiation. We provide evidence that TCFAP2C represses Nanog and activates Cdx2. These
results indicate that TE maintenance depends on the presence of both Cdx2 and Tcfap2c.

Several transcription factors such as Cdx2 and Tead4 were detected in murine preimplantation stages and were shown to be required for the specification of the trophoderm cell lineage (35, 40). Here, we demonstrate that Tefap2c is expressed in mouse embryos prior to implantation at the morula and blastocyst stage by RT-PCR analysis. We showed that the overexpression of Tefap2c in ESCs led to the induction of a TSC-like fate, as demonstrated by the upregulation of Cdx2 as well as other markers indicative of undifferentiated TSCs. These cells are stable in culture because they can be passaged for a prolonged time without the loss of undifferentiated TSC morphology. Also, they differentiate and become all of the derivatives of the TE, as demonstrated in vitro and in vivo.

Taken together, the results of these experiments indicate a role in TSC specification and maintenance. The following data argue against the specification scenario: (i) the TCFAP2C protein is downregulated in the ICM of late blastocysts only, when the TE is already specified, and (ii) mutants for Tcfap2c and Eomes arrest and die at about the blastocyst stage (5, 34). Tcfap2c−/− embryos are formed and implant into the uterus, indicating that Tcfap2c is not essential for TE specification. Tcfap2a/Tcfap2c double mutant blastocysts can be observed; therefore, a functional redundancy in TE specification between those two molecules can be ruled out as well (39). We favor the interpretation, that Tcfap2c has a subordinate role to Cdx2 and Eomes in specification of the TE.

Our interpretation suggests that Tcfap2c primarily plays a role in the maintenance of the TE. In support of this hypothesis, no permanent Tcfap2c-deficient TSC line (i.e., passage 10 and higher) was established. It is known that the expression of Elf5 in the TE creates a positive-feedback loop with Cdx2 and Eomes and reinforces the commitment to the trophoblast lineage (25). A lack of Elf5 expression renders the TSC labile and prone to differentiation. TSC-like cells generated by the overexpression of Tcfap2c express Elf5, suggesting the induction of the TSC maintenance program. Also, as mentioned above, the Tcfap2c-induced TSC-like cultures grow for an extended time as compact colonies that express the TSC marker CDH3, further strengthening the role of Tcfap2c in TSC maintenance.

Interestingly, the overexpression of Cdx2 in Tcfap2c−/− ESCs or the overexpression of Tcfap2c in Cdx2−/− ESCs led to the induction of TSC fate, but Elf5 was not expressed. The cells fail to establish morphologically distinct TSC colonies and initiate differentiation into TE derivatives. Therefore, we conclude that both CDX2 and TCFAP2C are required to transactivate Elf5, which in turn protects TSCs from differentiation.

This scenario is supported by in vivo data. For example, extraembryonic tissues that are in contact with the Fgf4-producing ICM of the blastocyst and the epiblast at the egg cylinder stage, namely, the polar TE (pTE) and the ExE, maintain TSCs (20, 36). Elf5-deficient embryos implant, but Cdx2 and Eomes expression cannot be maintained in the TSC compartment of the ExE; therefore, the embryos die (7). Similarly, the ExE of Tcfap2c-deficient mice at E7.5 lacks the expression of Cdx2 and Eomes (2). We speculate that the Tcfap2c mutants may have depleted their TSC population, resulting in reduced cell numbers in the ectoplacental cone, as well as reduced numbers of giant cells.

On the molecular level, we showed that TCFAP2C transactivated the TSC marker Cdx2 and repressed the pluripotency gene Nanog. Hence, Tcfap2c can be integrated in the framework of genes regulating TE fate, such as Gata3, Cdx2, Eomes, and Elf5. The concerted action of the repression of Oct3/4 and Nanog (6, 28) and the Tcfap2c-mediated repression of Nanog result in the promotion of TE fate. Both Cdx2 and Tcfap2c activate each other and are required to establish Elf5 expression. In addition, Tcfap2c and Cdx2 may transactivate an overlapping set of target genes to orchestrate TE lineage differentiation. In addition, Kidder and Palmer recently applied ChIP-on-chip analysis to TSCs and showed that TCFAP2C binds to the promoter of Gata3, Tead4, and Elf5, indicating a possible cross-regulation between the factors responsible for the specification and maintenance of TE fate (19).

Activation of the Ras-MAPK pathway in ESCs induced TSC-like cells, which resulted in the transcriptional upregulation of Tcfap2c and Cdx2 and a reduction in the Nanog protein level (21). We hypothesize that Ras-MAPK-induced repression of Nanog and the upregulation of Cdx2 may be mediated in part by TCFAP2C. Induction of the Ras-MAPK pathway in Tcfap2c-deficient ESCs should shed light on this molecular cascade. In addition to Tead4 (27), Wnt signaling is able to trigger Cdx2 expression and TE lineage differentiation in ESCs (14). Data from Xenopus show that Tcfap2c is also induced by Wnt signaling (42); therefore, expression of both Cdx2 and Tcfap2c may be initiated in response to Wnt signaling.

Why is Tcfap2c expression not restricted to pTE and ExE but can be observed in all TE derivatives except the syncytiotrophoblast cells during development? This occurrence may be due to an additional role for Tcfap2c in promoting proliferation/endoreduplication during differentiation. Data from transgenic mice show that the overexpression of Tcfap2c results in enhanced proliferation in mammary gland epithelial cells (18). We speculate that this role in supporting proliferation may also be required during differentiation of the TE lineage. In addition, the formation of the syncytiotrophoblast, which is negative for TCFAP2C, requires exit from the cell cycle, supporting this notion (17). Also, TCFAP2C levels are high in human cytotrophoblast cells, but TCFAP2C is downregulated upon terminal differentiation in vitro (31).

The schematic in Fig. 7 shows our current model. Tead4 activates Gata3 (30) and Cdx2 (25), both initiate Eomes expression, and Cdx2 represses Oct3/4 and Nanog, Oct3/4, Sox2, and Nanog stimulate each other (4), and Nanog and Oct3/4 repress Cdx2 (6, 28). Cdx2 and Elf5 induce each other, leading to the fixation of the TE lineage (25). Tcfap2c and Cdx2 activate each other, and TCFAP2C represses Nanog. Both TCFAP2C and CDX2 are required to establish Elf5 expression, and the expression of Elf5 leads to the stabilization of CDX2 levels (25). Together, the expression of these factors maintains the pool of self-renewing TSCs in the trophoderm, the ExE, and the ectoplacental cone (Fig. 7B, green boxes). During induction of differentiation in the chorion, the labyrinth, trophoblast giant cells, and the spongiotrophoblast, the expression of Cdx2 and, thereafter, the expression of Elf5 are lost. In this situation, TCFAP2C supports proliferation/endoreduplication in the TE derivatives (Fig. 7B, yellow boxes). Tcfap2c must be downregulated before cell cycle exit can occur, which is required for
syncytiotrophoblast differentiation (Fig. 7B, red box). Elucidation of the programs regulated by Tcfap2c in the TE lineage will help in deciphering the genes governing TE maintenance and differentiation.

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FIG. 7. (A) Model integrating Tcfap2c in the network of transcription factors governing TEC specification and maintenance. (B) Schematic view of TEC differentiation. Green boxes, self-renewing TSCs are present; Cdx2, Elf5, and Tcfap2c are expressed; yellow and green box, TEC derivatives where Elf5 and Tcfap2c expression can be detected; yellow boxes, TEC derivatives that show Tcfap2c expression but that lack Cdx2 and Elf5; red box, Tcfap2c-negative, proliferation-inactive cells of the syncytiotrophoblast.

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