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The dual roles of geminin during trophoblast proliferation and differentiation

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ABSTRACT

Geminin is a protein involved in both DNA replication and cell fate acquisition. Although it is essential for mammalian preimplantation development, its role remains unclear. In one study, ablation of the geminin gene (*Gmnn*) in mouse preimplantation embryos resulted in apoptosis, suggesting that geminin prevents DNA re-replication, whereas in another study it resulted in differentiation of blastomeres into trophoblast giant cells (TGCs), suggesting that geminin regulates trophoblast specification and differentiation. Other studies concluded that trophoblast differentiation into TGCs is regulated by fibroblast growth factor-4 (FGF4), and that geminin is required to maintain endocycles. Here we show that ablation of *Gmnn* in trophoblast stem cells (TSCs) proliferating in the presence of FGF4 closely mimics the events triggered by FGF4 deprivation: arrest of cell proliferation, formation of giant cells, excessive DNA replication in the absence of DNA damage and apoptosis, and changes in gene expression that include loss of Chk1 with up-regulation of p57 and p21. Moreover, FGF4 deprivation of TSCs reduces geminin to a basal level that is required for maintaining endocycles in TGCs. Thus, geminin acts both like a component of the FGF4 signal transduction pathway that governs trophoblast proliferation and differentiation, and geminin is required to maintain endocycles.

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Introduction

Despite the numerous targeted mutations generated in mice, only 25 or so zygotically expressed genes have been shown to be essential for the development of a fertilized egg into a blastocyst (Kohn et al., 2011). One of these is geminin, a small protein originally identified in *Xenopus* embryos as both an inhibitor of DNA replication (McGarry and Kirschner, 1998) and an inducer of neural plate expansion (Kroll et al., 1998). Geminin plays different roles during the development of multicellular animals. Geminin prevents premature loading of the replicative MCM helicase onto replication origins ((Kisielewska and Blow, 2012; Klotz-Noack et al., 2012) and references therein), and geminin influences cell fate acquisition by associating with transcription factors or chromatin modifying proteins ((Caronna et al., 2013; Lim et al., 2011) and references therein). Thus, it is not surprising that attempts to identify the role of geminin in preimplantation mammalian development have proven enigmatic.

Preimplantation development begins with fertilization and ends with implantation of the resulting blastocyst. A blastocyst consists of a monolayer of epithelial trophoblast cells (trophectoderm) that envelop the remaining pluripotent blastomeres (inner cell mass (ICM)) within a cavity (blastocoel). Although ablation of the geminin gene (*Gmnn*) in mice arrested development as early as the 4-cell to 8-cell stage in two independent studies, the phenotypes differed significantly. In one study (Hara et al., 2006), *Gmnn*-/- embryos appeared to lack a trophectoderm (although trophoblast biomarkers were not analyzed), and the blastomeres embryos underwent DNA replication in the absence of mitosis, sustained DNA damage, and entered apoptosis. This study was consistent with the established role of geminin in preventing 'DNA re-replication', an aberrant event in which cells undergoing mitotic cell divisions initiate a second S-phase before the first S-phase is completed. DNA re-replication results in DNA damage, induction of the DNA damage response, and apoptosis (Ding and MacAlpine, 2010; Kerns et al., 2012; Klotz-Noack et al., 2012; Yanagi et al., 2005; Zhu and Depamphilis, 2009).

In a separate study (Gonzalez et al., 2006), all of the blastomeres in *Gmnn*-/- embryos appeared to differentiate into the trophoblast giant cells (TGCs) that are essential for implantation of the blastocyst and subsequent placentation. These embryos contained a blastocoel cavity but lacked an ICM, expressed genes characteristic of TGCs, and over-replicated their nuclear DNA. Implied, but not proven, was that *Gmnn* ablation induced

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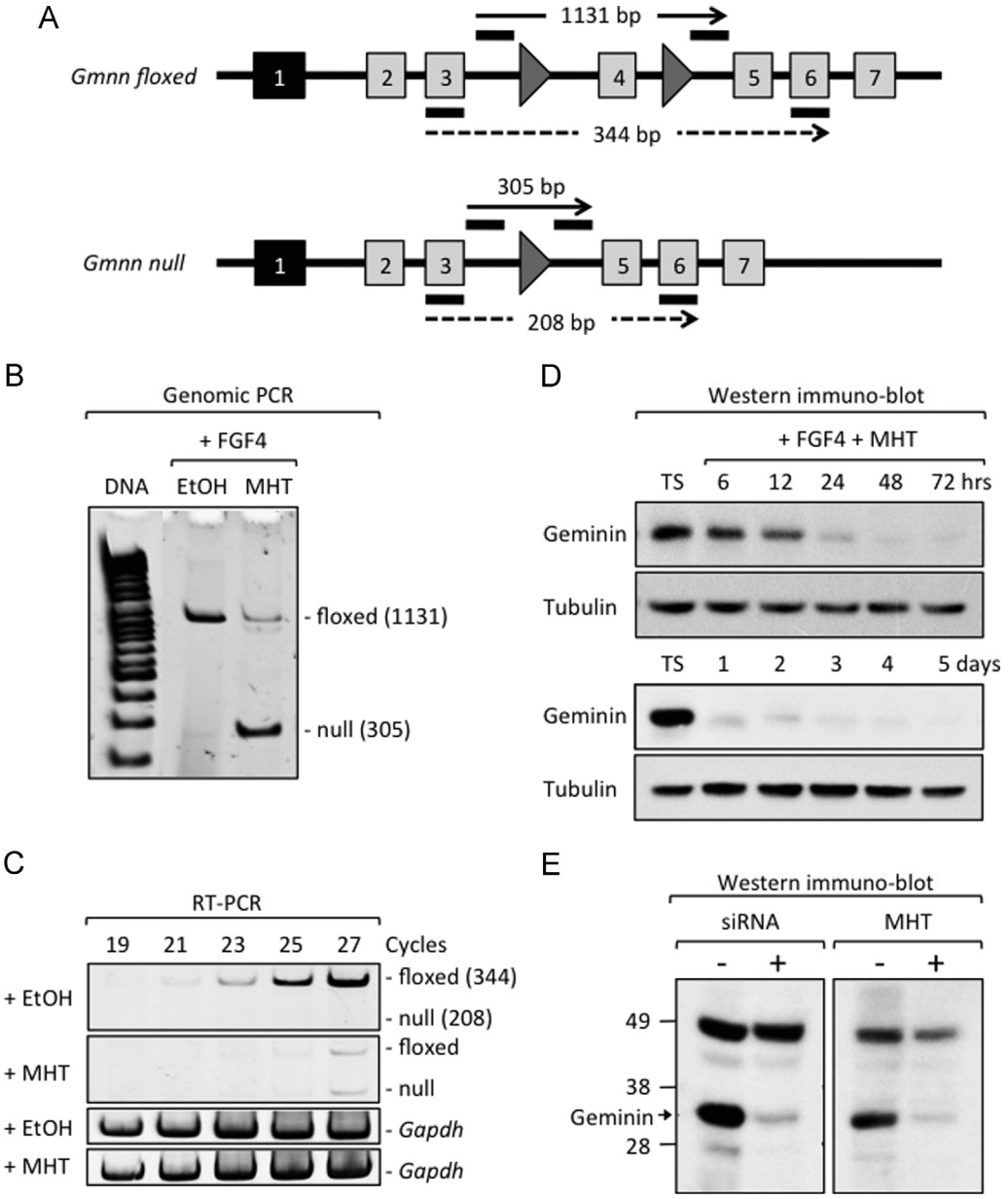

Fig. 1. Conditional knockout of *Gmnn* exon 4 in TSCs depletes geminin mRNA and protein. (A) Schematic representation of *Gmnn* floxed allele and null allele after recombination by Cre-recombinase. Black and gray boxes represent non-coding and coding exons, respectively. Exon 4 is flanked by two LoxP sites (triangles) and is deleted after recombination by Cre recombinase. Solid boxes above and below the gene represent PCR primers used for genomic PCR and RT-PCR, respectively, with the expected fragment sizes for genomic PCR indicated by solid arrowed lines and for RT-PCR indicated by dashed arrowed lines. (B) $Gmnn^{flox/flox}$, *ER-Cre*/+ TSCs were treated for 3 days with either 3 μM MHT or the equivalent amount of the ethanol vehicle. On day 5, DNA was extracted for genomic PCR. The floxed allele amplicon is 1131 bp. The null allele amplicon is 305 bp. (C) $Gmnn^{flox/flox}$, *ER-Cre*/+ TSCs were treated as in panel B, but total RNA was extracted for RT-PCR. Glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) provided a loading control. The floxed allele amplicon is 344 bp. The null allele amplicon is 208 bp. (D) $Gmnn^{flox/flox}$, *ER-Cre*/+ TSCs were treated with 3 μM MHT for the indicated times. Whole-cell extracts were subjected to Western immuno-blotting to detect geminin levels. Tubulin provided a loading control. (E) Geminin protein was detected by Western immuno-blotting based on its molecular size, its sensitivity to anti-*Gmnn* siRNA in HCT116 cells as described in (Zhu and Depamphilis, 2009), and its sensitivity to ablation of the geminin gene in $Gmnn^{flox/flox}$, *ER-Cre*/+ TSCs.

endoreplication, a hallmark of TGCs. Endoreplication (also termed endoreduplication) refers to the occurrence of a second S-phase in the absence of an intervening mitosis and cytokinesis (reviewed in (Zielke et al., 2013)). Multiple S-phases in the absence of mitosis and cytokinesis are referred to as 'endocycles'. Cells that are developmentally programmed for endoreplication, such as TGCs, do not undergo apoptosis, but form viable, nonproliferating, mononuclear, polyploid cells. These results suggested that down-regulation of *Gmnn* expression induces differentiation of pluripotent blastomeres into trophectoderm and eventually into TGCs. Subsequent studies in which *Gmnn* expression was suppressed in P19 embryonal carcinoma cells and embryonic stem cells suggest that high levels of geminin sustain the expression of genes that prevent differentiation of pluripotent cells into trophoblasts (Yang et al., 2011, 2012).

This conclusion, however, is difficult to reconcile with the fact that differentiation of trophoblasts into TGCs first occurs in peri-implantation blastocysts in response to the absence of fibroblast growth factor-4 (FGF4, (Arman et al., 1999; Keramari et al., 2010; Murohashi et al., 2010; Nichols et al., 1998; Xu et al., 1998)) that is produced by the ICM (Roberts and Fisher, 2011). Moreover, trophoblast stem cells (TSCs) differentiate into TGCs when deprived of FGF4 (Simmons and Cross, 2005; Tanaka et al., 1998), and these TGCs continue to express geminin (Ullah et al.,