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Alterations in the spatiotemporal expression pattern of geminin during human epidermal morphogenesis

Efstathia Pasmatzi[1] ✉, Alexandra Monastirli[1], Zoi Lygerou[2], Stavros Taraviras[3], Stavros Kakkos[4], George Stamatiou[5], Dionysios Tsambaos[1]

[1]Department of Dermatology, School of Medicine, University of Patras, Rio-Patras, Greece. [2]Department of Biology, School of Medicine, University of Patras, Rio-Patras, Greece. [3]Department of Physiology, School of Medicine, University of Patras, Rio-Patras, Greece. [4]Department of Vascular Surgery, School of Medicine, University of Patras, Rio-Patras, Greece. [5]Department of Gynecology and Obstetrics, Iasso General Hospital, Athens, Greece.

Abstract

Introduction: Geminin, a (25 kDa) protein, was originally identified as a key regulator of DNA replication licensing in the cell cycle and of cell fate during embryonic nervous system formation. Although geminin is involved in mechanisms underlying the regulation of transcription and patterning in embryonic development, its expression and possible significance in human epidermal morphogenesis remains unknown.
Methods: Forty-one skin biopsy specimens obtained from human fetuses (10th to 23rd week of estimated gestational age) were processed for immunohistochemistry using a primary rabbit polyclonal antibody against geminin.
Results: Distinct and statistically significant qualitative and quantitative alterations in the spatiotemporal expression pattern of geminin were observed in the developing human epidermis.
Conclusions: The highly ordered expression of geminin in different layers of fetal human epidermis reported here for the first time suggests that this protein may play a significant role in epidermal morphogenesis. However, the mechanisms underlying the alterations of the geminin expression pattern during fetal development at the molecular level remain to be elucidated. Further studies are now warranted to address whether the expression pattern of geminin in the developing human epidermis is disturbed in fetuses with genodermatoses and whether these disturbances might be important for prenatal diagnosis of genodermatoses.

Keywords: geminin, embryogenesis, keratinocytes, human embryonic epidermis, estimated gestational age

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Introduction

Geminin is a small (~25 kDa) protein consisting of about 209 amino acids that is encoded by the *GMNN* gene (located on chromosome 6) and is predominantly localized in the nucleus of diverse cycling cell populations. Geminin is structurally characterized by a central coiled-coil domain, through which geminin homodimerizes and interacts with cell cycle regulators (1–5). Geminin was originally identified as a bifunctional protein with roles in both the maintenance of genome integrity by means of regulation of the fidelity of DNA replication licensing and in the control of cell fate during nervous system embryogenesis (6, 7).

During a cell cycle, geminin accumulates in S phase, subsequent to the initiation of DNA replication. It then binds to Cdt1, a key member of the pre-replication complex, inhibits its association with replication origins, and prevents the recruitment of licensing factors such as the minichromosome maintenance (MCM) complex onto origins during S, G2, and early M phases. Thus, it inhibits re-replication and ensures replication once per cell cycle and genomic stability (8). Expression of geminin increases through the late phases of the cell cycle and reaches its maximum level in mitosis. Then, geminin is downregulated by ubiquitin-dependent proteolysis through the anaphase, promoting complex/cyclosome (APC/C) (7, 9, 10).

Interestingly, although geminin is generally believed to safeguard the genome, accumulating evidence suggests that this protein interacts with an ever-growing number of partner proteins playing a central integrative role in multiple cellular events (11), including carcinogenesis. Indeed, it has been shown that suppression of geminin activity leads to DNA re-replication and apoptosis in cancer cells (12) and promotes cellular senescence (13). Geminin ablation enhances tumorigenesis in mice (14), and overexpression of geminin stimulates cell cycle progression and proliferation in normal and cancer tissues promoting tumor invasion and metastasis and markedly affects cancer prognosis (15–18), whereas a compound targeting geminin was recently shown to promote DNA damage and cell death in cancer cells (19).

Although geminin is involved in various stages of embryonic development playing a significant role in the mechanisms underlying the regulation of transcription and patterning factors (20–24), surprisingly little is known about the expression and possible significance of this multifunctional protein in human epidermal morphogenesis. Thus, this article investigates the expression pattern of geminin in the developing epidermis and uses immunohistochemistry to define the sequence of its spatiotemporal alterations in human fetuses from the 10th to the 23rd week of estimated gestational age (EGA).

✉ Corresponding author: efipas@upatras.gr; pasmatzi@otenet.gr; pasmatzi@med.upatras.gr
**Dedicated to the memory of Professor Demetrios Kelekis, Department of Interventional Radiology, School of Medicine, Universities of Patras and Athens, Greece.*

Methods

Tissue samples

On March 23rd, 1998, the local ethics committee of Iasso Hospital approved the protocol of this study on the expression pattern of diverse proteins (including geminin) during morphogenesis of human epidermis, in accordance with the ethical standards laid down in an appropriate version of the 1964 Declaration of Helsinki, as revised in 1983. The Maternity and Gynecology Clinic provided biopsy skin specimens obtained from the tibiae of morphologically normal human embryos following legal abortions performed at this institution. The ages of the fetuses were 10 weeks EGA (n = 8), 12 weeks EGA (n = 7), 14 weeks EGA (n = 6), 16 weeks EGA (n = 6), 18 weeks EGA (n = 8), and 23 weeks EGA (n = 6), and they were confirmed not to have a family history of cutaneous disorders. EGA was determined from maternal histories, fetal measurements (crown, rump, and foot length), and comparative histological appearance of the epidermis (25–28). All specimens were fixed in buffered formalin (10%) and embedded in paraffin. Part of the 4 μm sections was routinely stained with hematoxylin and eosin and examined histologically, and the other part was processed for geminin immunohistochemistry.

Immunohistochemistry

The expression of geminin was investigated by immunohistochemistry in formalin-fixed and paraffin-embedded skin biopsy specimens (4 μm sections) using a primary rabbit polyclonal antibody against geminin (kindly supplied by Zoi Lygerou) at a dilution of 1:500, and the peroxidase-labeled streptavidin-biotin standard technique (29). The staining was graded independently by two experienced dermatopathologists, and the results of their evaluation were classified on a scale of 0 to 3 as follows: 0 = negative stain, 1 = weak intensity, 2 = moderate intensity, 3 = strong intensity.

Statistical analysis

The results of this study were statistically analyzed with the Jonckheere–Terpstra test (30, 31) and the Wilcoxon rank sum test (32) using the software product SAS® (SAS Institute, Cary, NC, USA), version 8.02. The level of significance was fixed at α = 5%. A p value < 0.05 was considered to indicate statistical significance.

Results

Immunohistochemistry

The results of the immunohistochemical investigation of fetal human epidermis (EGA: 10–23 weeks) using an antibody against geminin are shown in Table 1. Interestingly, in all age groups the basement membrane of the fetal skin revealed no geminin immunoreactivity.

10th and 12th week estimated gestational age

Because the findings in the epidermis of fetuses aged 10 and 12 weeks EGA were almost identical, they are described here together. In all specimens, the cells of the periderm and the intermediate layer revealed no geminin immunoreactivity, whereas those of the basal layer revealed moderate to strong cytoplasmic and nuclear expression of geminin (Fig. 1A).

14th week estimated gestational age

In all specimens, the cells of the periderm and the basal layer revealed moderate cytoplasmic geminin expression, whereas those of the intermediate layer were devoid of any geminin immunoreactivity (Fig. 1B).

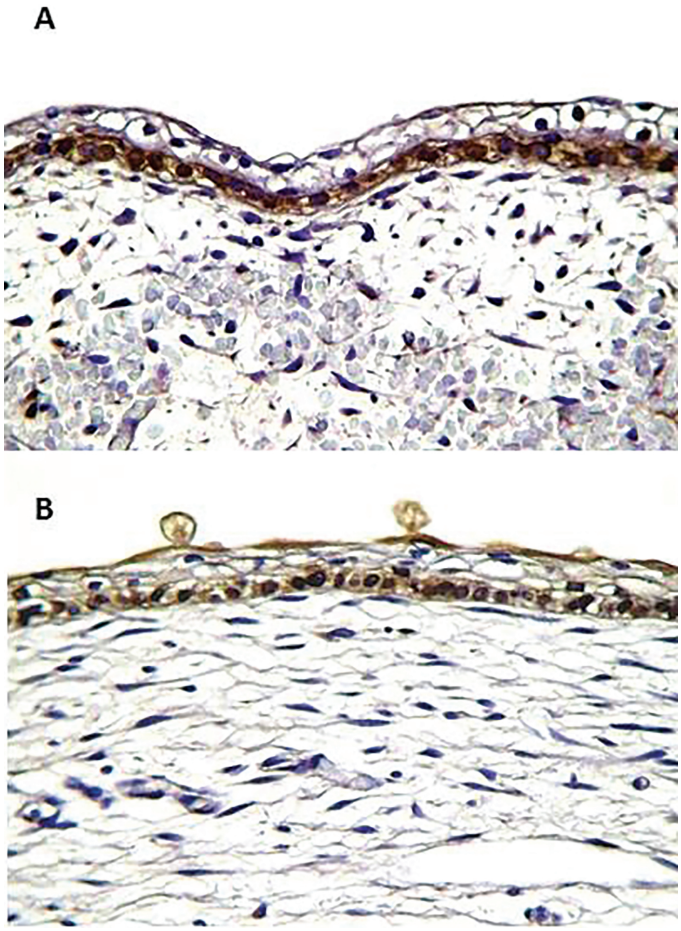

Figure 1 | A) Expression pattern of geminin in human fetal epidermis of gestational age 10 weeks. In contrast to the cells of the periderm and the intermediate layer, which reveal no immunoreactivity, those of the basal layer showed moderate to strong cytoplasmic and nuclear expression of geminin; B) expression pattern of geminin in human fetal epidermis of gestational age 14 weeks. The cells of the periderm and the basal layer reveal moderate cytoplasmic geminin expression, whereas those of the intermediate layer are devoid of any geminin immunoreactivity.

Table 1 | **Results of immunohistochemical staining of fetal human epidermis (gestational age: 10–23 weeks) with antibody against geminin.**

		10 weeks (n = 8)	12 weeks (n = 11)	14 weeks (n = 9)	16 weeks (n = 8)	18 weeks (n = 10)		23 weeks (n = 6)
Gem	E	0 1 2 3	0 1 2 3	0 1 2 3	0 1 2 3	0 1 2 3		0 1 2 3
							HL	6 0 0 0
	PD	8 0 0 0	11 0 0 0	0 0 9 0	0 5 3 0	0 2 5 3	GL	0 2 4 0
	IL	8 0 0 0	11 0 0 0	9 0 0 0	0 0 4 4	0 0 6 4	SL	0 1 3 2
	BL	0 0 3 5	0 0 4 7	0 0 9 0	8 0 0 0	10 0 0 0	BL	6 0 0 0

Gem = geminin, E = epidermis, PD = periderm, IL = intermediate layer, BL = basal layer, HL = horny layer, GL = granular layer, SL = spinous layer.

16th week estimated gestational age

In all specimens, weak to moderate and moderate to strong cytoplasmic geminin immunoreactivity was observed in the cells of the periderm and the intermediate layer, respectively, whereas the cells of the basal layer revealed no geminin expression (Fig. 2A).

18th week estimated gestational age

In all specimens, the cells of the periderm and the intermediate layer revealed weak to strong and moderate to strong cytoplasmic geminin immunoreactivity, respectively, whereas those of the basal layer were devoid of any geminin expression.

23rd week estimated gestational age

In all specimens, no geminin immunoreactivity was seen in the cells of the horny and basal layers. The cells of the granular and spinous layers revealed weak to moderate and weak to strong cytoplasmic geminin immunoreactivity, respectively (Fig. 2B).

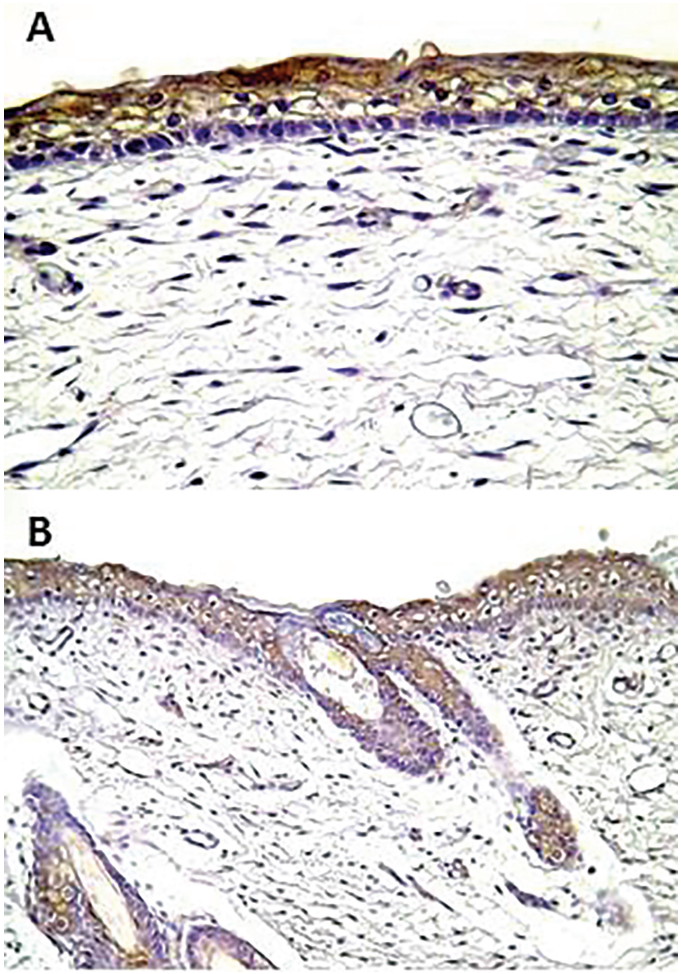

Figure 2 | A) Expression pattern of geminin in human fetal epidermis of gestational age 16 weeks. Weak to moderate and moderate to strong cytoplasmic geminin immunoreactivity is shown in the cells of the periderm and the intermediate layer, respectively. The cells of the basal layer are devoid of any geminin expression; B) expression pattern of geminin in human fetal epidermis of gestational age 23 weeks. In contrast to the cells of the horny and basal layer, which show no geminin immunoreactivity, those of the granular and the spinous layer reveal weak to moderate and weak to strong geminin expression, respectively.

Statistical analysis

The independent-samples analysis examined in this article with the Jonckheere–Terpstra test for ordered alternatives was performed to investigate overall associations. In cases of significance, we performed multiple tests for the various subgroups with significance values adjusted by the Bonferroni correction. The statistically significant differences between the age groups studied with regard to the intensity of geminin immunoreactivity of the layers of fetal human epidermis can be summarized as follows.

Periderm: statistically significant differences in the intensity of cytoplasmic immunoreactivity of periderm were found between specimens of the 10th and 14th week EGA ($p < 0.001$), 10th and 16th week EGA ($p = 0.002$), 12th and 18th week EGA ($p < 0.001$), and 14th and 16th week EGA ($p = 0.046$).

Intermediate layer: statistically significant differences in the intensity of cytoplasmic immunoreactivity of the intermediate layer were found between specimens of the 10th and 16th week EGA ($p = 0.002$), 10th and 18th week EGA ($p = 0.001$), 12th and 16th week EGA ($p < 0.001$), 12th and 18th week EGA ($p < 0.001$), 14th and 16th week EGA ($p = 0.001$), and 14th and 18th week EGA ($p = 0.001$).

Basal layer: statistically significant differences in the intensity of cytoplasmic immunoreactivity of the basal layer were found between specimens of the 10th and 16th week EGA ($p = 0.003$), 10th and 18th week EGA ($p = 0.001$), 10th and 23rd week EGA ($p = 0.009$), 12th and 14th week EGA ($p = 0.040$), 12th and 16th week EGA ($p = 0.001$), 12th and 18th week EGA ($p < 0.001$), 12th and 23rd week EGA ($p = 0.004$), 14th and 16th week EGA ($p = 0.001$), 14th and 18th week EGA ($p < 0.001$), and 14th and 23rd week EGA ($p = 0.002$).

Discussion

Morphogenesis of human epidermis is a fundamental biological process implicating an integrated network of signaling pathways and mechanical forces that interfere with each other and lead to a well-orchestrated progressive transformation of the embryonic single epithelial cell layer covered by the periderm into a stratified epithelium and finally into the fully keratinized epidermis. Investigations on the developing human epidermis are of particular importance because they may lead to a better understanding of the mechanisms underlying epidermal morphogenesis at the molecular and cellular level and possibly contribute to the advancement of prenatal diagnosis of inherited disorders of keratinization (33).

Epidermal morphogenesis can be divided into the embryonic period (until the 8th week EGA) and the fetal period (from the 9th week EGA). The latter is subdivided into the epidermal stratification period (9th to 14th week EGA) and the interfollicular keratinization period (14th to 24th week EGA). Human epidermis initially consists of a single layer of cuboid basal epithelial cells. The periderm is formed during the 4th week EGA and is located immediately above the basal layer of the epidermis. Around the 9th week EGA, the intermediate cell layer is formed below the periderm; thus, from the 10th to the 20th week EGA fetal epidermis consists of three distinct epithelial layers: the periderm, the intermediate layer, and the basal layer. At the 23rd week EGA, the periderm is sloughed from the epidermal surface into the amniotic fluid and

the fetal epidermis consists of four distinct epidermal layers: the horny, granular, spinous, and basal layers (34).

The spatiotemporal expression pattern of geminin occurring in human fetal epidermis with advancing gestational age is reported here for the first time. In the specimens of the 10th and 12th week EGA, the basal layer of developing epidermis reveals moderate to strong geminin nuclear and cytoplasmic immunoreactivity, whereas the periderm and the intermediate layer are completely devoid of any expression of geminin. In the specimens of the 14th week EGA, moderate cytoplasmic immunoreactivity for geminin is present in both the basal layer and the periderm, whereas the intermediate layer still shows a negative immune reaction to geminin. In the specimens of the 16th and the 18th week EGA, moderate to strong expression of geminin appears for the first time in the intermediate layer. The periderm shows weak to strong geminin immunoreactivity, whereas the basal layer reveals for the first time negative immunoreactivity to geminin. Finally, in the specimens of the 23rd week EGA there is geminin immunoreactivity in the granular layer (weak to moderate) and in the spinous layer (weak to strong), whereas the basal and horny layers are completely devoid of any geminin expression.

The highly ordered expression of geminin in different layers of fetal human epidermis reported here suggests that this protein may play a significant role in epidermal morphogenesis. However, the mechanisms underlying the alterations of the geminin expression pattern during fetal development at the molecular level remain to be elucidated. During embryonic development, pathways that regulate cell cycle progression, acquisition of cell fate, and cellular differentiation must be temporally and spatially regulated and strictly coordinated in the embryonic tissues, processes in which geminin may play a significant role. Indeed, recent accumulating evidence indicates that this protein, apart from being a key regulator of DNA replication licensing in the cell cycle and a transcriptional molecular switch determining cell fate, reveals a wide spectrum of additional activities including binding to and interference with the activities of homeodomain-containing transcription factors, inhibition of diverse protein activities, interactions with regulators of chromatin structure, and cellular transition from proliferation to differentiation (17, 22, 35, 36).

The sequence of changes in the expression of geminin observed in this study particularly in the basal layer during the early stages of human epidermal morphogenesis is a feature not shared by other fetal human epithelia and merits particular attention. Indeed, in the specimens of the 10th and 12th week EGA, geminin immunoreactivity of basal cells was localized in both the nucleus and the cytoplasm, whereas in the specimens of the 14th week EGA geminin expression in the nucleus was completely lost and immunoreactivity to geminin was exclusively localized in the cytoplasm of basal cells. Interestingly enough, in the subsequent stages of epidermal morphogenesis (16th, 18th, and 23rd week EGA) cytoplasmic expression in basal cells also disappeared. It is worth noting that in all layers of human fetal epidermis but the basal one, geminin expression was confined to the cytoplasm of epidermal cells. Because geminin is known to strongly correlate with undifferentiated and proliferating cells in fetal and adult tissues (9), the complete lack of geminin expression in the basal cells is a striking and unexpected feature of fetal epidermis after the 14th week EGA, which remains unexplained to us.

Geminin has originally been described as a nuclear protein, and many of its biological activities are known to occur in the nucleus (6, 7, 37). Interestingly, cytoplasmic localization of geminin has been observed in cells that reveal nuclear membrane breakdown as well as in post-mitotic cells that lack periods of nuclear membrane disassembly (38, 39). It therefore seems reasonable to suggest that geminin is capable of shuttling between subcellular compartments with its translocation and activity being regulated by complex mechanisms involving distinct protein motifs.

The results of this immunohistochemical study, which was performed in a large number of fetal skin specimens, clearly show distinct qualitative and quantitative alterations in the spatiotemporal expression pattern of geminin during epidermal morphogenesis prior to keratinization. The mechanisms underlying these alterations at the molecular level remain to be elucidated. Given the central role of geminin in proliferation/differentiation decisions in other tissues, such as the nervous and hematopoietic system (11), it would be interesting to assess the expression pattern of geminin in the epidermis of fetuses with genodermatoses, which often exhibits proliferation/differentiation abnormalities. It would also be of interest to extend this study to other samples, such as the oral mucosa, given earlier correlations of geminin expression levels with oral cancers.

Conclusions

This study shows that the expression of geminin in human fetal epidermis, reported here for the first time, is characterized by a highly ordered pattern and distinct qualitative and quantitative alterations during epidermal morphogenesis prior to keratinization, suggesting that this multifunctional protein may play an important role in epidermal morphogenesis. Further studies are now warranted to unravel the underlying mechanisms at the molecular level and to address whether the expression pattern of geminin in the developing human epidermis is disturbed in fetuses with genodermatoses.

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