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Fetal Cell Microchimerism; Normal and Immunocompromised Gestations in Mice

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ABSTRACT

Objective: To compare fetal cell microchimerism in normal and immunocompromised gestations. **Materials and methods:** The study consists of two groups of mature female mice. In the control group and the immunocompromised study group, 5 mg of saline and cyclosporine were injected intraperitoneally, respectively. In the second step, all female mice were mated with "Actine-Luc (+) green fluorescent protein (GFP)" transgenic male mice. Immunohistochemical studies (ALPL-anti-luciferase, cytokeratin-antiluciferase, and CD 105-antiluciferase) were carried out on maternal liver, skin, and lung tissues at 6–7th and 14–15th gestational days, and postpartum 3–4th, 12th, and 18–24 months. **Results:** GFP (+) cells were detected in maternal liver and skin but not in lung tissue. Liver was the most affected tissue. GFP was found to be more intense in the immunocompromised group. **Conclusion:** Fetal microchimerism was demonstrated in maternal liver and skin and found to be more intensive in the immunocompromised group.

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Pregnancy; fetal cell microchimerism; immunohistochemistry; cyclosporin

Introduction

Fetal cell microchimerism refers to the persistence of fetal cells in the maternal tissue following pregnancy [1, 2]. Fetal cells have been found to engraft in various tissues in both humans and animal models [3, 4]. These engrafted cells may demonstrate the capacity of multidirectional differentiation and may result in different types of biological events [5–7]. However, the triggering mechanisms behind the differentiation of these engrafted fetal cells are still obscure and not properly defined.

Fetal microchimeric cells may differentiate along the hematopoietic pathway [8, 9]. Studies also showed that engrafted fetal cells may differentiate to the host tissue cells in

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case of various types of injuries, especially in liver and kidneys [5, 10]. It has also been reported that fetal cells may engraft in tissues like the skin and lung [11, 12]. Skin engraftment is very important because there are conflicting findings in this field. Skin engraftment of fetal cells may be associated with various immunological skin disorders such as systemic sclerosis and may take a positive role in wound healing [13, 14]. Fetal microchimerism may be the biological rationale behind some autoimmune disorders.

There are also some publications related to the role of fetal cell microchimerism in the pathophysiology of various obstetric complications such as recurrent miscarriage and preeclampsia [15, 16]. Fetal cell microchimerism and cancer is another topic to be studied [17, 18].

Another important issue in fetal cell microchimerism is the immunological background of pregnancy in humans and animals such as mice. We believe that the maternal immune system is at the center of the events related to fetal cell engraftment and the differentiation of engrafted cells in various biological pathways. This study demonstrates the presence of engrafted fetal cells in the liver, skin, and lungs of the female mice that gave birth at the end of normal and immunocompromised gestational conditions.

Materials and methods

The study consists of two groups of mature female mice (wild-type Swiss Albino mice). In the control group (n:8), 5 mg of saline were injected intraperitoneally for 5 days, while 5 mg of cyclosporine (Sandimmun 50 mg, Novartis) was also injected intraperitoneally for 5 days in the immunocompromised study group (n:8).

In the second step of the study, these two groups of female mice mated with “Actine-Luc (+) green fluorescent protein (GFP)” transgenic male mice. Transgenic mice were kindly donated from Prof. Dr. Med. Vet. Jörg Haberstroh, Freiburg, Germany (FVB/N-Tg(β-Actin-luc)-Xen, Common Name: β-Actin-luc, Genotype: Hemizygous, Background Strain FVB/N, Coat Color: white).

Pregnant mice from both groups were evaluated at 6–7th and the 14–15th days of their pregnancies. In vivo imaging of transgenic fetuses was performed by IVIS three-dimensional bioluminescence/fluorescence optical imaging system IVRS50 (Xenogen Alameda). Five minutes after 150 mg/kg luciferase injection into the peritoneum, mice were sedated with sebofluorein and placed in the “black box” revealing the transgenic fetuses visible to the IVS 100 camera. Captured images by the camera were transferred to the computer to be used for the evaluation. Two female mice were decapitated (under general anesthesia) at the 6–7th and 14–15th days (one mouse from each group) of their pregnancies. GFP (+) fetuses and maternal organs were carefully obtained and kept at suitable conditions to be used for immunohistochemical examinations in the future.

The remaining female mice of the study groups (six mice in each group) were kept separated after their deliveries for microchimerism studies. Then, two mice from each group were decapitated under general anesthesia 4 months after delivery. The organs of the animals were carefully preserved for further analysis and immunohistochemical studies. In this study, we have used liver, skin, and lungs to show fetal microchimerism.

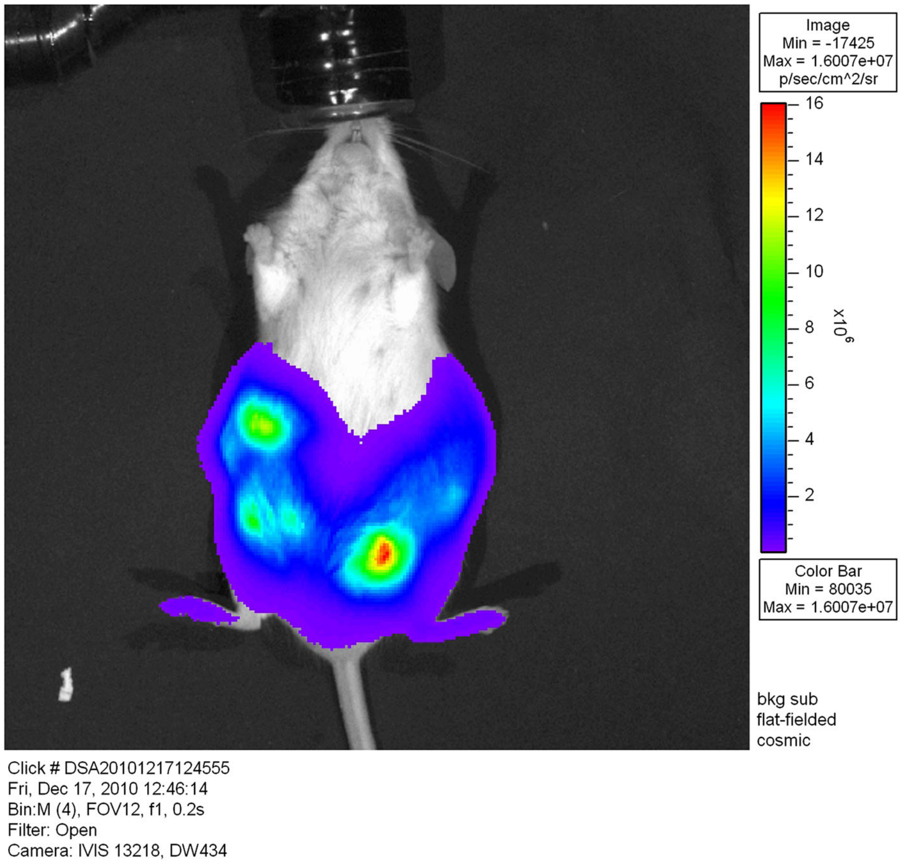

Figure 1. Pregnant non-transgenic mouse with transgenic fetuses (14th gestational day).

This process was repeated 12 and 24 months after the deliveries of the mice. Only one mouse from the immunocompromised group survived until the end of the second year. All animal procedures were performed in accordance with the "Guidelines for the Care and Use of Experimental Animals." This study was approved by "Hacettepe University Research Animals Ethics Committee" (2007/6-2).

Tissues obtained from the sacrificed animals were immediately frozen in liquid nitrogen. Five micrometer thick sections were taken from the tissues. Cytokeratin-antiluciferase (1/100-1/100; MyBioSource, San Diego, USA, Cat. No: MBS247528/Cat. No: MBS534493), ALPL-antiluciferase (1/100-1/100; Abbiotec, San Diego, USA, Cat. No: 251825/MyBioSource, San Diego, USA, Cat. No: MBS534493) were used as primary antibodies. FITC (MyBioSource, San Diego, USA, Cat. No: MBS538496) and Texas red (MyBioSource, San Diego, USA, Cat. No: MBS538623) were the fluorescence labeled secondary antibodies. DAPI (Biolegend, London, UK, Cat. No: 422801) was used for the nuclei. Sections were evaluated using a Leica 6000B microscope. Images were captured with a Leica DC490 (Wetzlar-Germany) digital camera.

ALPL-antiluciferase, cytokeratin-antiluciferase were used during the course of immunohistochemical studies to stain the tissues. In this study, only the photographs of double-stained tissues were used. The levels of staining were classified as +, ++, and +++

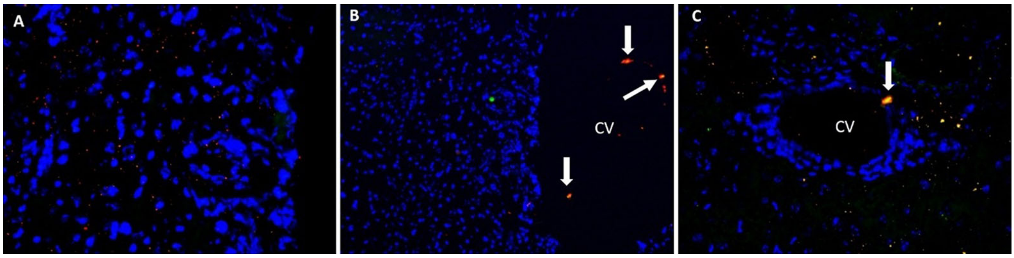

Figure 2. Liver: ALPL-anti-luciferase staining. (a) Control: female non-pregnant mouse; (b) 6–7th gestational day mouse; (c) 14th gestational day mouse. Thick white arrows: anti ALPL-anti luciferarase/double-positive cells, CV: central vein (a–c: Texas red + FITC + DAPI, ×40).

according to staining density heuristically by two different experts in a double-blind manner.

Results

Figure 1 shows in vivo imaging of transgenic fetuses (14th gestational day) by IVIS 3-dimensional bioluminescence/fluorescence optical imaging system IVRS50 (Xenogen Alameda).

In this study, we used maternal liver, skin, and lung tissues to demonstrate fetal microchimerism at the 6–7th and 14–15th gestational days, and 4 months, 12 months, and 24 months after delivery. GFP (+) cells, which were presumably from transgenic fetuses, were detected in liver and skin but not in lung tissues by double immunohistochemical staining at the times studied.

Figure 2 shows the liver tissues of control, and 6–7th and 14–15th gestational-day mice. Antiluciferase/ALPL positive, double-stained cells were observed to increase in number in 14–15th days when compared to 6–7th gestational days. Double-stained (GFP + and ALPL +) cells are observed in the endothelium (Figure 2c) and in the parenchyme (Figure 2b) of the liver of the pregnant mice. The liver seems to be the most affected tissue among the studied tissues in terms of fetal microchimerism. We have observed no difference between immunosuppressed mice and normal mice at 6–7th and 14–15th gestational days.

GFP/ALPL double-positive cells were present in the postpartum period in both normal and immunocompromised pregnant animals. In the fourth month, they were more numerous in the immunocompromised group. Similarly, double-positive cells appeared to be more numerous in the immunocompromised group at 12 and 24 months in a decreasing manner, respectively (Figure 3). Table 1 summarizes these findings.

For skin analysis, we have used the ear tissues of the mice. Anti-cytokeratin was used in immunohistochemical studies. However, we could not preserve ear tissues of six to seven gestational days due to technical difficulties.

The immunohistochemical findings were almost similar to each other in the control and study group during pregnancy (14–15 days). Cytokeratin positive cells were mostly observed in the hair follicles and in the cartilage. GFP (+) cells were observed at the basal layers of the epidermis in both groups. Few cells were stained in the cartilage but none in the hair follicles in both groups (Figure 4a, b). In 14–15 days pregnant group,

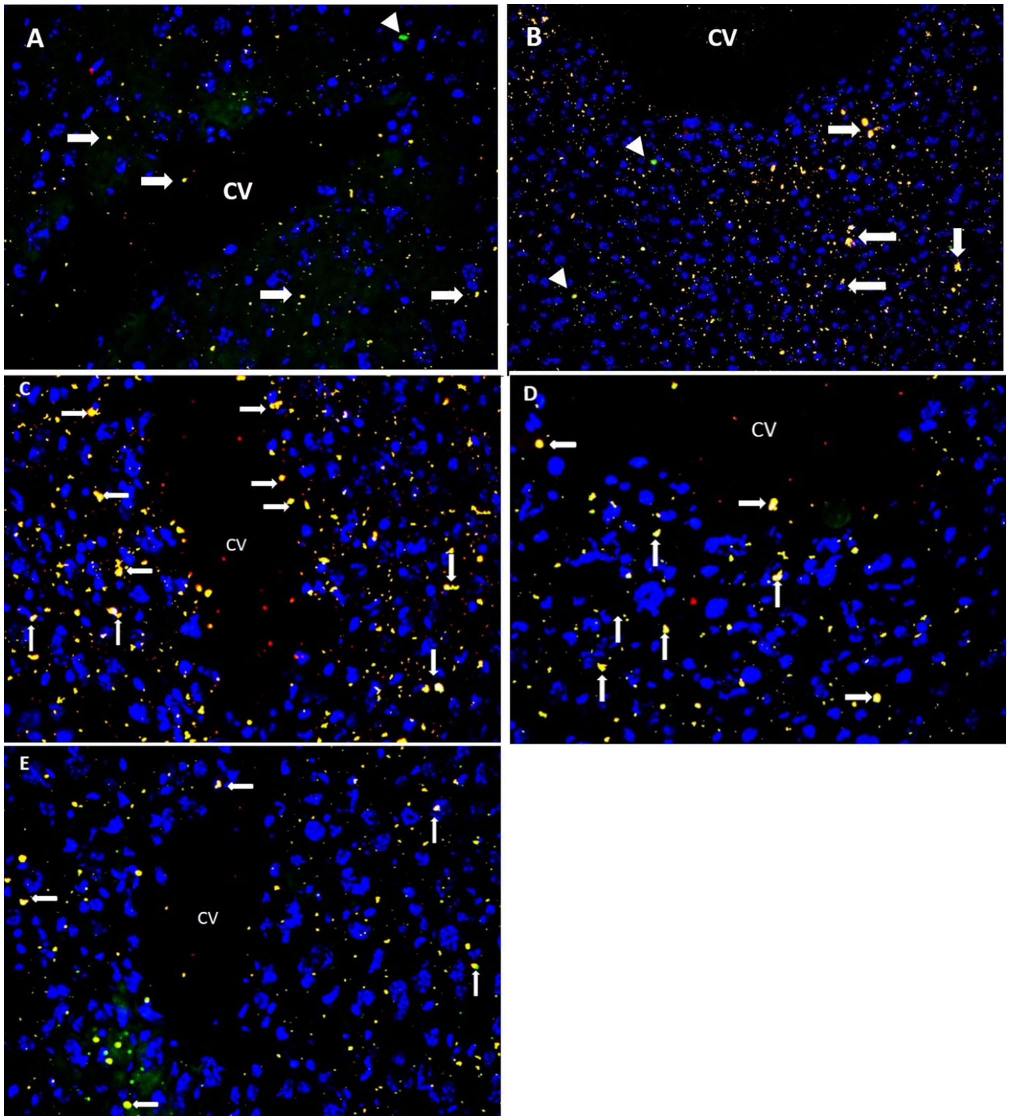

Figure 3. Liver: anti ALPL-antiluciferase. (a) Four months normal; (b) four months immunosuppressed; (c) one year normal; (d) one year immunosuppressed; (e) two years immunosuppressed. (a–e) Thick white arrows: anti ALPL-anti-luciferarase/double-positive cells. White arrow heads: luciferase positive cells. CV: central vein (a–e: Texas red + FITC + DAPI, ×40).

with double staining, GFP/anti-cytokeratin positive cells were observed in the dermis and few in the cartilage (Figure 4b).

GFP positive cells are observed in epidermis, dermis, and in the cartilage, but not in hair follicles both in normal and immunosuppressive groups at the postpartum fourth month. Anti-cytokeratin cells were observed in epidermis, dermis, cartilage, and hair follicles in both groups. In epidermis and dermis, they were more numerous in the immunosuppressive group. Anti-cytokeratin-anti GFP, double-positive cells were observed in epidermis, dermis, and in the cartilage, but not in hair follicles (Figure 5a, b).

Table 1. GFP, ALPL, and double staining of the liver paranchyme at normal and immunosuppressive rats.

Liver Group	Parenchyme GFP	ALPL	D
Control	+	++	−
6–7th days N	+	++	+
6–7th days IS	+	++	+
14–15th days N	+	++	+
14–15th days IS	+	++	+
4 months N	++	++	++
4 months IS	++$^{+}$	++$^{+}$	++$^{+}$
12 months N	+++	+++	+++
12 months IS	+++$^{+}$	+++$^{+}$	+++$^{+}$
24 months N	N/A	N/A	N/A
24 months IS	+	++	+

N: normal; IS: immunosuppressed; GFP: green fluorescent protein; ALPL: alkaline phosphatase; D: double; N/A: non-applicable; $^{+}$: more numerous.

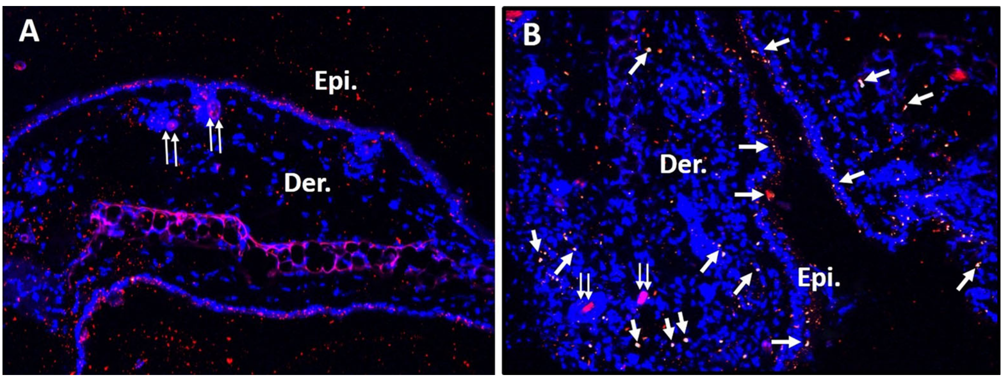

Figure 4. Skin: anti cytokeratin – anti luciferase. (a) Control: female non-pregnant mouse; (b) 14–15 days pregnant mouse. Thick white arrows: anti-cytokeratin – anti-luciferarase/double-positive cells. Double thin arrows: only anti-cytokeratin positive hair follicles. Epi: epidermis, Der: dermis (a, b:Texas red + FITC + DAPI, ×40).

At the end of the first year, anti-GFP, anti-cytokeratin, anti-GFP/anti-cytokeratin, and double-positive cell distribution were almost similar to the four-month group. But, as a difference, in the immunosuppressive group, anti-GFP positive cells were observed in the hair follicles which were absent in the control group. Double-positive cells were also seen in the hair follicles. Double-stained cells were more numerous in the normal group than the immunosuppressive group.

Only one animal in the immunosuppressive group survived for 2 years. The distribution of anti-GFP, anti-cytokeratin, and double-stained cells were almost similar to the 14–15 days pregnant mouse except the presence of double-stained cells in the hair follicles similar to the one-year immunosuppressive group. Table 2 summarizes these findings.

We could not demonstrate significant GFP positivity in the lung tissues of either the control group or the immunocompromised group in our study.

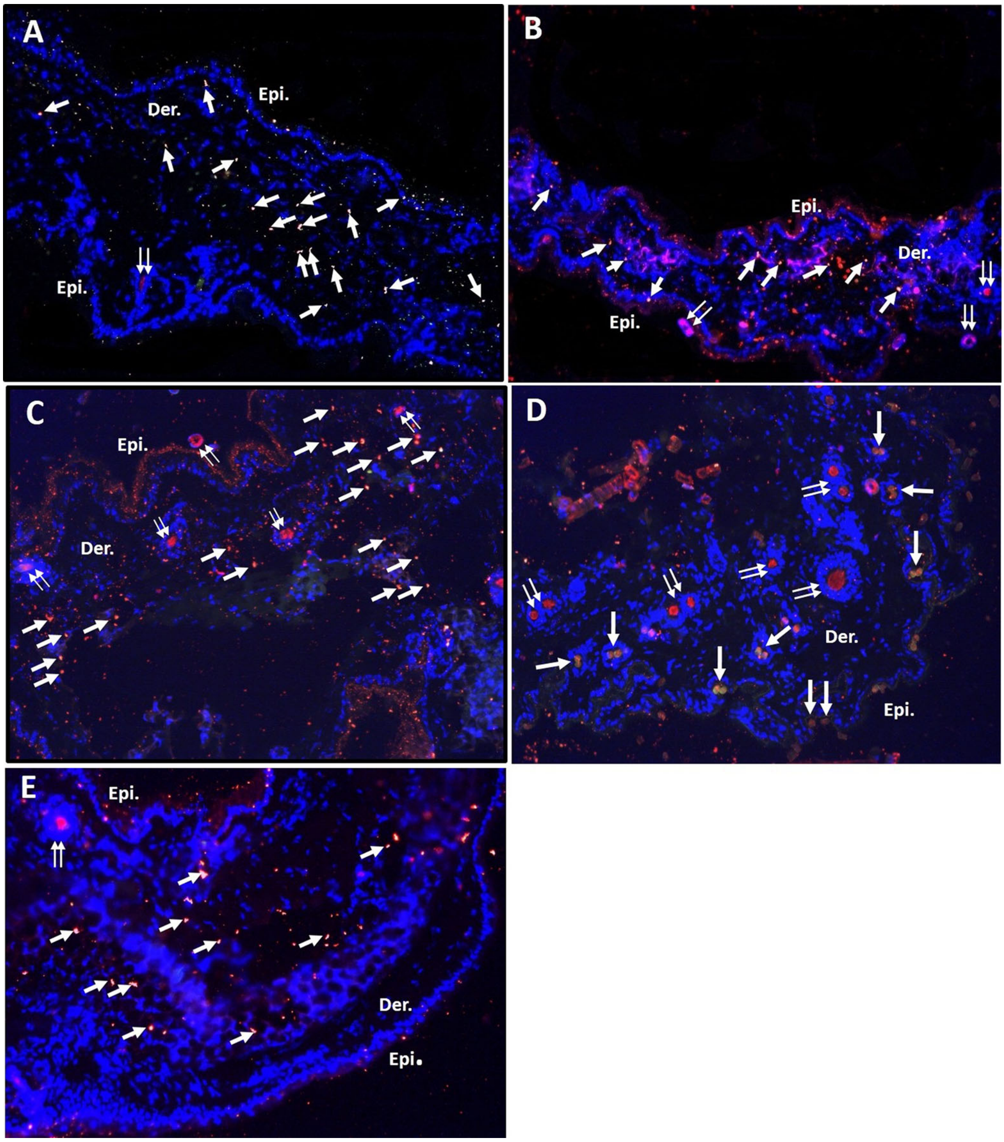

Figure 5. Skin: anti-cytokeratin – anti-luciferase. (a) Four months normal; (b) four months immunosuppressed; (c) one year normal; (d) one year immunosuppressed; (e) two years immunosuppressed. (a–e) Thick white arrows: anti-cytokeratin-anti luciferase/double-positive cells. Double thin arrows: only anti-cytokeratin positive hair follicle. Epi: epidermis, Der: dermis (a–e: Texas red + FITC + DAPI, ×40).

Discussion

Fetal cell microchimerism is defined as the persistence of fetal cells in maternal organs and circulation without any apparent graft vs. host reaction or graft rejection. The main question is the nature of fetal microchimerism (under physiological conditions vs. at the presence of pathophysiological disorders) and the outcomes of this immune system related biological event. Thus, there are still several queries to be answered, such as the "type/nature" of fetal cells, timing of the presence of these cells in the maternal

Table 2. GFP, ALPL, and double staining of the skin at normal and immunosuppressive rats.

SKIN	Epidermis			Dermis			Hair follicles			Cartilage		
Group	GFP	CK	D	GFP	CK	D	GFP	CK	D	GFP	CK	D
N	−	+++	−	−	+	−	−	++	−	−	++	−
6–7th days	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø
14–15th days	+	++	+	+	+	+	−	++	−	−	+	−
4 months N	++	++	++	++	++	++	−	++	−	+	+	+
4 months IS	++	+++	++	++	+++	++	−	++	−	+	+	+
12 months N	+++	++	+++	+++	++	+++	−	++	−	+	+	+
12 months IS	+++	++	+++	+++	++	+++	++	++	++	+	+	+
24 months N	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
24 months IS	+	++	+	+	+	+	−	+	−	+	+	+

N: normal; IS: immunosuppressed; GFP: green fluorescent protein; CK: cytokeratin; D: double; N/A: non-applicable.

circulation, factors affecting the amount of these cells and associated fetal DNA, reaction of maternal innate and humeral immune systems, the mechanisms of cellular settlement at the maternal tissues, and short- and long-term advantages vs. disadvantages of microchimerism on women's health [19].

The origin of fetal cells, especially stem cells, is still to be determined in normal and complicated pregnancies. The fetal cell transfer pathways involved in normal pregnancies most likely are influenced by various maternal or fetal risk factors affecting the cellular structures of the maternal-fetal interface of the placenta where fetal perfusion takes place [4]. Here is demonstrated the importance of physiological apoptosis of cyto/syncytiotrophoblastic cells and aponecrotic changes due to placental inflammation as a result of associated maternal health disorders (metabolic, immunologic, chronic inflammatory, infectious, toxic, etc.). These situations most likely affect the timing of the transfer as well as the amount of cells and associated fetal DNA. Maternal health status, especially the epigenetic factors and immune system disorders, may also be responsible from the engraftment of placental stem cells and the cleavage of the other mature fetus originated cells [20].

There are various publications related to the timing and pathway type of fetal microchimerism and the maternal tissues involved in these processes [11]. Circulating fetus originated cells and engrafted fetal stem cells may have clinical implications on the type of future biological processes which influence maternal health. Liver, skin, kidneys, hematopoietic system, and lungs are the main targets for the engraftment of fetal stem cells [21]. The other important inquiry is the nature of fetus originated cells and their fate, and the mechanisms involved in the conversion of placental stem cells to host tissue cells. Adaptation mechanisms of these converted stem cells to the host tissue are also the concern of current studies. The importance of short- and long-term clinical signs and effects of the elimination of fetal DNA together with non-stem cells and their degradation products must also be investigated to ellucidate the engraftment processes of placental stem cells. There are many animal studies to reveal the impact of fetal microchimerism on women's health [5, 7, 22].

This study uses a transgenic mice model to demonstrate fetal microchimerism during pregnancy and the postpartum period in normal and immunocompromised mice by immunohistochemical examinations of maternal liver, skin, and lung tissues. We have shown fetal microchimerism during pregnancy and after pregnancy in liver and skin tissues of mice. We could not show fetal microchimerism in the lung tissues because of lack of engraftment or due to technical reasons. We also show that microchimerism is more obvious later in pregnancy. The engraftment profile in liver and skin tissue increases until the postpartum 12th month and exists at the end of the second year in a decreasing manner. We have shown that engrafted stem cells were present in the liver and the hair follicle structures.

We have designed our study to demonstrate difference between immunocompromised and normal pregnant mice in terms of density of fetal cell microchimerism. Our results demonstrated increased density of fetal microchimerism which may have many clinical results. Fetal cell microchimerism is related with pregnancy complications such as preeclampsia which has complicated etiological pathophysiological pathways [15, 23]. Autoimmune disorders such as scleroderma are also associated with increased amounts of fetal cell microchimerism [24]. Thus, our results have great clinical importance as patients with immunosuppression may have a greater risk of pregnancy complications or long-term maternal health problems via increased fetal microchimerism. Nevertheless, our results must be confirmed via prospective human studies for clinical application.

Fetal microchimerism was demonstrated in maternal liver and skin. It was found to be more intensive in the immunocompromised group. We believe that further investigation is necessary to understand the impact of fetal microchimerism on obstetric outcomes and maternal health.

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