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RĪGAS STRADIŅA UNIVERSITĀTE

Anna Miskova

APPEARANCE OF GROWTH FACTORS, GENES AND THEIR PRODUCTS IN CASES OF HUMAN EMBRYO TUBAL AND INTRAUTERINE IMPLANTATION

Summary of Doctoral Thesis
Speciality – Morphology

Riga, 2012

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Summary of the promotional work

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The Doctoral Thesis was developed at the Department of Morphology of the Institute of Anatomy and Anthropology of the Riga Stradins University

Scientific supervisors:

Dr. habil. med., professor **Mara Pilmane** AAI RSU

Dr. med., assoc. professor **Dace Rezeberga** Department of Obstetrics and Gynaecology RSU

Approved reviewers:

Dr. med., assoc. professor **Ilze Štrumfa** RSU

Dr. med. vet., assoc. professor **Ilze Matise-Van Houtan** VMF LLU

Dr. med., **Kristiina Rull** Tartu University (Estonia)

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Dr. habil. med., professor **Līga Aberberga-Augškalne**

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TEXT ABBREVIATIONS

Abbreviation	English definition	Latvian definition
AFP	alpha-fetoprotein	alfa-fetoproteīns
BARX1	barx1 homeobox gene	homeoboksa barx1 gēns
bFGF	basic fibroblast growth factor	bāziskais fibroblastu augšanas factors
cAMP	cyclic adenosine monophosphate	cikliskais adenozīnamonofosfāts
CB	canabioid (endogenous)	kanabioīds (endogēnais)
CNS	central nerve system	centrālā nervu sistēma
Conceptus	the product of the union of oocyte and spermatozoon at any stage of development including	ieligzdenis
DNA	deoxyribonucleic acid	dezoksiribonukleīnskābe
EGF	epidermal growth factor	epidermālais augšanas faktors
FGFR1	fibroblast growth factor receptor1	fibroblastu augšanas faktora 1 receptors
FSH	follicle stimulating hormone	folikulu stimulējošais hormons
GFAP	glial fibrillary acidic protein	glijas fibrillārais skābais proteīns
HB-EGF	heparin binding epidermal growth factor	heparīnu piesaistošais augšanas faktors
HGF	hepatocyte growth factor	hepatocītu augšanas faktors
hCG	human chorionic gonadotropine	cilvēka horiona gonadotropīns
HOXA10	homeobox protein a 10	*homeobox* olbaltumviela 10
hPL	human placental lactogen	cilvēka placentārais laktogēns
ICC	interstitial cells of cajal	intersticiālās kajala šūnas
IGF-1	insulin-like growth factor 1	insulīnam līdzīgais augšanas faktors 1
IGF-1R	insulin-like growth factor 1 receptor	insulīnam līdzīgā augšanas faktora 1 receptors
IGFBP-1	insulin-like growth factor 1 binding protein	insulīnam līdzīgā augšanas faktora 1 piesaistes olbaltumviela
PID	pelvic inflammatory disease	iegurņa iekaisuma slimība
IL	interleukin	interleikīns

the end of the table

Abbreviation	English definition	Latvian definition
iNOS	inducible nitric oxide synthesis	inducējama slāpekļa oksīda sintēze
IUD	intrauterine device	intrauterīna spirāle
IVF	*in vitro* fertilization	ārpusdzemdes apaugļošana
LIF	leukemia inhibiting factor	leikēmiju inhibējošais faktors
MMP	matrix metalloproteinase	matrices metaloproteināze
Msx2	muscle segment homeobox 2 gene	muskuļu segmentu homeoboksa gēns 2
NGF	basic nerve growth factor	bāziskais nervu augšanas faktors
NGFR	nerve growth factor receptor	nervu augšanas faktora receptors
PAPP-1	pregnancy associated paraproteine a	ar grūtniecību saistītais paraproteīns a
PDGF	platelet derived growth factors	trombocītu atvasinātais augšanas faktors
PGP 9.5	protein gene product 9.5	proteīngēnviela 9.5
PNS	peripheral nerve system	perifērā nervu sistēma
PROKR	prokinetocine receptor	prokinetocīnu receptors
Sox17	sry (sex determining region y)-box 17 gene	sox17 (dzimumnoteicošā rajona) gēns
STD	sexually transmitted disease	seksuāli transmisīva saslimšana
TGF beta 1	transforming growth factor-beta 1	transformējošais augšanas faktors beta 1
TNF alfa	tumour necrosis factor alpha	audzēja nekrozes faktors alfa
TUNEL	terminal deoxynucleotidyl transferase mediated dutp nick end labeling	deoksinukleotidiltransferāzes mediēta dns terminālo 3`- galu marķēšana ar deoksiuridinfosfātu
VEGF	vascular endothelial growth factor	vaskulārais endotēlija augšanas faktors

TOPICALITY OF THE PROMOTIONAL WORK

Embryo implantation is a complicated process involving mother and *conceptus* cells differentiation, proliferation and invasion that are essential for successful pregnancy. Normally developed embryo at blastocyst stage and receptive endometrium are necessary for successful implantation. This process is dependent on molecular interaction between mother and embryo cells. Approximately only 25-30% of fertilized human eggs continue development till live birth (Macklon et al., 2002). Nearly 30% of embryo implantation loss are due to unreceptive endometrium (Chard, 1991), but 1-2% of human embryo implantations are ectopic (Farquar, 2005; Varma et Gupta, 2009). It is still unknown which of factors are involved in human embryo implantation process.

World Health Organization (WHO) declared, that approximately 1 of 250 human embryo implantations are ectopic (approximately 7 implantations per hour), which is a threatening condition for mother's health and her life (Varma and Gupta, 2009).

Usually a woman`s reproductive health and fertility decreases after having an ectopic pregnancy. Early diagnosis of ectopic pregnancy is essential for successful treatment. That is why it is important to understand morphopathogenesis of ectopic pregnancy.

Growth factors control proliferation, migration and differentiation of cells, but appropriate gene expression regulates embryo development (Attar, 2004). Probably implantation site and further embryo development are dependent on mother to embryo molecular interactions. It is established, that human decidual cells at implantation site express IGF-1, but IGF-1 knockout mice are infertile (Kapur et al., 1992; Baker et al., 1996).

There are lack of data on growth and transcriptional factor distribution in ectopic and uterine implantation tissue. The importance of growth factors in ectopic pregnancy pathogenesis and clinical use are not well understood.

The aim of this study was to analyze the role of growth factors, their receptors, BARX1 and Msx2 distribution in human embryo, endometrial and oviduct tissues in a case of uterine or tubal pregnancy and to establish the role of these factors in tubal pregnancy pathogenesis.

HYPOTHESIS OF THE PROMOTIONAL WORK

1. Human embryo implantation site depends on local growth factors, their receptors and some gene distribution and expression
2. Specific growth factor and their receptor distribution is found in tubal tissue in a case of tubal implantation

OBJECTIVES OF THE PROMOTIONAL WORK

1) to obtain human embryo tissue the development of which was terminated by legal abortion before 12 gestational weeks;
2) to obtain tubal tissue in tubal pregnancy operation material;
3) to obtain endometrial tissue in surgical abortion operation material;
4) to perform morphologic description of routine slides;
5) to detect growth factors' immuhistochemical distribution;
6) to detect mesenchyma inducting genes and transcription factors; expression in early stages of embryonal development;
7) to use TUNEL method for detection of apoptotic cells in different human embryo tissues;
8) to perform statistical analysis on growth factors and some gene expression correlation in pathologic blastocyst implantation site.

NOVELTY OF THE PROMOTIONAL WORK

Growth factors, their receptors, Msx2, BARX1 immunochemical detection and apoptosis analysis were done in endometrial and 5/6-12 gestational week's embryo tissues for the first time.

Growth factors and their receptor immunochemical detection and apoptosis analysis were done in tubal pregnancy tissue for the first time.

The importance of IGF-1, IGF-1R, FGFR1, bFGF, NGF, NGFRp75, PGP 9.5, HGF, human defensin beta 2, MMP 9 un MMP 2 was shown in tubal pregnancy.

The distribution of human defensin beta 2 was described in 5/6-12 gestational weeks human embryo tissue.

THE STRUCTURE AND VOLUME OF THE DOCTORAL THESIS

The thesis for PhD degree is written in Latvian. The promotion work consists of 8 parts: introduction, review of literature, materials and methods, results, discussion, conclusions, the list of references and the appendix. The volume of the promotion work comprises 183 pages, including 39 tables and 3 figures, 92 slides. The list of references consists of 413 titles. There are 19 publications on the topic of the promotion work thesis.

MATERIALS AND METHODS

Three groups of human tissue have been included in the study:

1. oviduct tissue taken from tubal pregnancy patients;
2. surgical pregnancy operation material;
3. embryo tissue taken from surgical pregnancy operation material.

The study was performed with the permission of Ethical Committee of Riga Stradins University (18.12.2007).

Human oviduct parts were obtained from 17 patients of Riga 1st Hospital, who had underwent salpingoectomy for tubal pregnancy with informed consent. Patients treated surgically, by means of salpingectomy were included in the study. Both conventional and endoscopic operational methods were used, depending on technical possibility.

Human embryo and gravid endometrium tissue were obtained from 10 patients who had had termination of unplanned pregnancy in Riga Medical Centre "Elite" with informed consent. Pregnancy termination was done surgically by uterus curettage. Embryo tissue was found in operation material: two complete human embryos and human embryo parts, such as extremities, internal organ complex and a head.

Tissue samples were taken from January 2007 till January 2008. Age, parity, contraception method, the number of pelvic inflammatory and sexually transmitted disease episodes and partner had been carried out for all patients.

The tissue samples were fixed in 2% formaldehyde and 0.2% picric acid mixture with 0.1 M phosphate buffer (ph 7.2). Then the samples were rinsed in thyroid buffer containing 10% sucrose and embedded in paraffin. The tissues were cut into 6-μm-thick sections and were dewaxed with toluene and rehydrated through a graded ethanol series. The sections were stained with

haematoxylin and eosin (H&E) using standard procedures to obtain a review picture of the slide.

We used biotin–streptavidin method for determination of growth factors, their receptors, BARX1, Msx2, human defensin beta 2 and MMPs. Growth factor, receptors and genes involved in the study are summarized in table 1.

The TUNEL method was applied for determination of apoptosis in the tissues using in situ cell death detection kit (POD Cat. No. 11 684 817 910 Roche Diagnostic).

At least five microscopic fields (X200) were analyzed using the microscope Leica DM RB (Leica Microsystems, Germany).

The distribution of these factors was detected semi quantitatively: 0/- occasional positive structure in visual field, + few positive structures in visual field, ++ moderate number of positive structures in visual field, +++ numerous positive structures in visual field.

For the group description we used the generally accepted descriptive statistical methods (Altman, 1991; Altman, 2000; Teibe, 2007).The data were analyzed by nonparametric rank analysis with SPSS Statistic 17 software. A Mann-Whitney U test was used as appropriate for evaluation of significant

For comparison of a number of samples we used the Kruskal Wallis test. The correlation coefficient *r* as a quantitative indicator of coherence tightness between two or more variables calculated for the ranking values (Spearman's Rank Correlation Coefficient). In the study the qualitative coherence tightness between variables, on the grounds of the correlation coefficient value, was assessed as small, medium or strong. The distribution of the correlation coefficient was as follows: $r = 0 - 0.3$, small, insignificant correlation; $r = 0.4 - 0.7$, medium correlation; $r = 0.7 - 0.9$, strong correlation.

Table 1

Data on antibodies applied in immunohistochemistry

Factor	Code	Obtained from	Dilution	Manufacturer
BARX1	ab26156	rabbit	1:250	*Abcam*, UK
bFGF	ab16828	rabbit	1:200	*Abcam*, UK
Human defensin beta 2	AF2758	goat	1:100	*RnDsystems*, Germany
CK13	ab58744	rabbit	1:250	*Abcam*, UK
CK5	ab24647	rabbit	1:1000	*Abcam*, UK
FGFR1	ab10646	rabbit	1:100	*Abcam*, UK
GFAP	M0761	mouse	1:50	*DakoCytomation*, Denmmark
HGF	AF-294-NA	goat	1:300	*RnDsystems*, Germany
Chromogranin A and B	B315100	mouse	1:10	*DakoCytomation*, Denmmark
IGF-1	MAB291	goat	1:100	*RnDsystems*, Germany
IGF-1R	AF-305-NA	mouse	1:100	*RnDsystems*, Germany
Caspase 6	ab52951	mouse	1:100	*Abcam*, UK
MMP 2	AF902	goat	1:100	*RnDsystems*, Germany
MMP 9	AF911	goat	1:250	*RnDsystems*, Germany
Msx2/HOX8	ab22601	mouse	1:250	*Abcam*, UK
NGF basic	ab6199	rabbit	1:500	*Abcam*, UK
NGFRp75	M3507	mouse	1:150	*DakoCytomation*, Denmmark
PGP 9.5	Z 5116	rabbit	1:150	*DakoCytomation*, Denmmark
Synaptophysin	M0776	mouse	1:10	*DakoCytomation*, Denmmark
TGF beta 1	ab1279	mouse	1:1000	*Abcam*, UK

RESULTS

Morphologic description of tubal pregnancy

Routine H&O slides showed marked branched oedemic mucosal folds and massive haemorrhages. We have found proliferation of basal epitheliocytes in some cases. Tubal *lamina propria* demonstrated different variety of lymphocytes, leukocytes and macrophages infiltration as well as blood capillary stasis. Peripheral trophoblast cells were found in oviduct connective tissue. H&O slides exhibited chorionic villi ingrown into the tubal wall and fibrinoid sediment. Ingrown chorionic villi reached *tunica muclularis*, which locally was thin and atrophic.

Embryonic origin tissue such as secondary trophoblast villi ingrown into tubal wall or embryo membranes, were discovered in tubal lumen. There were no embryoblast derived tissues found in tubal pregnancy operational material.

Morphologic description of intrauterine pregnancy

Typical pregnancy changes such as widened branched crypts covered with fine cuboidal epitheliocytes were seen in H&O endometrium slides. Peripheral trophoblast cells, granulocytes and macrophages were seen in *decidua compacta*. Spiral arteries were formed in *decidua*. There were no differences in morphologic picture between the slides.

Chorionic villi, embryo membranes and different fragments of embryonic organs were discovered in all samples, and testified the intrauterine pregnancy.

Morphologic description of embryo tissue

Chorionic villi and embryonic membranes fragments were found in each sample. Different parts of embryo body were seen in each intrauterine pregnancy slide.

We have got two embryos and eight embryo body fragments. Embryonic development stage varies from 5/6 embryonic development week to 12 embryonic development week or from Carnegie 18 to Carnegie 23 stage.

H&O slides demonstrated embryonic CNS elements, such as spinal cord, brain fragments and spinal ganglia. We have obtained internal organ – heart, lungs, liver and *mesonephros* or *metanephros* fragments from nine embryos. Embryonic skeletal system: extremities, vertebra and rib fragments demonstrate the process of endochondral ossification.

Almost all embryonic organ – CNS, internal organ, extremities – structure corresponded to gestational stage, and testified the normal embryonic development.

Appearance of growth factors, their receptors, MMPs, human defensin beta 2 and apoptosis in tubal pregnancy tissue

Transforming growth factor beta 1: we have not found any TGF beta 1 positive cells in tubal pregnancy tissue.

Basic fibroblast growth factor and its receptor 1: we discovered few bFGF positive cells in all tubal tissues. Some epitheliocyte, endotheliocyte, mesotheliocyte *cytolemma* and miocyte *sarcolemma* contained bFGF. Some bFGF positive cells such as macrophages, neutrophils and peripheral trophoblast cells were seen in tubal *lamina propria*. There were no bFGF

positive cells in cytotrophoblast, syncytiotrophoblast and extraembryonic mesenchyma.

Moderate or numerous FGFR1 positive structures in tubal and trophoblast tissue were discovered. Tubal epithelium and smooth musculature contained numerous FGFR1 positive structures. Blood vessel wall and mesothelium accommodated moderate number of FGFR1 positive cells. Tubal *lamina propria* showed few FGFR1 positive macrophages and neutrophils. Peripheral trophoblast also demonstrated few FGFR1 positive cells.

Embryonic tissue such as syncytiotrophoblast and cytotrophoblast demonstrated moderate, but extraembryonal mesenchyma – a numerous number of FGFR1 positive structures.

Basic nerve growth factor and its receptor p75: NGF was found in all oviduct structures. There was a moderate number of NGF positive structures in tubal epithelium, smooth musculature, and endothelium and nerve fibres. Mesothelium, extraembryonal mesenchyma and peripheral trophoblast contained few NGF positive cells. Numerous cytotrophoblast cells contained NGF, but in syncytiotrophoblast NGF was absent. NGFRp75 was accommodated in oviduct *lamina propria* and *tunica muscularis* nerve fibres only.

Insulin like growth factor and its receptor: a numerous number of IGF-1 positive structures was seen in tubal epithelium, but IGF-1R contained only some of epitheliocytes. Despite epithelium moderate number of IGF 1R positive cells was discovered only in mesothelium.

Macrophages and neutrophils in tubal *lamina propria* contained IGF-1. Cytotrophoblast and syncytiotrophoblast accommodated a moderate number of IGF-1 positive cells. Some cells in extraembryonic mesenchyma and peripheral trophoblast were IGF-1 positive.

Hepatocyte growth factor: tubal epithelium contained numerous HGF positive cells. Blood vessel wall and smooth musculature accommodated

numerous positive HGF structures, as well as peripheral trophoblast, syncytiotrophoblast and cytotrophoblast.

Extraembryonal mesenchyma demonstrated a moderate number of HGF positive cells.

Tubal innervation: protein gene product 9.5: we have found numerous PGP 9.5 positive cells in tubal epithelium. PGP 9.5 positive neuroepithelial bodies were also seen in tubal epithelium. PGP 9.5 positive nerve fibres were located in oviduct connective tissue. Numerous PGP 9.5 positive cells were discovered in peripheral trophoblast. Tubal *lamina propria* demonstrated a moderate number of PGP 9.5 positive macrophages and neurophils.

Tissue degeneration enzymes in tubal pregnancy: we discovered numerous MMP 2 positive structures in tubal epithelium, endothelium, smooth musculature, nerve fibres and mesothelium. Tubal connective tissue had numerous MMP 2 positive macrophages and neutrophils. Cytotrophoblast, syncytiotrophoblast and extraembryonic mesenchyma accommodated numerous MMP 2 positive cells.

MMP 9 was found in numerous cells of oviduct epithelium, but endothelium and connective tissue contained a moderate number of MMP 9 positive structures. Smooth musculature, mesothelium and nerve fibres demonstrated few MMP 9 positive cells. Cytotrophoblast, syncytiotrophoblast and peripheral trophoblast contained moderate number, but extraembryonal mesenchyma few MMP 9 positive structures.

Programmed cell death in tubal pregnancy: tubal epithelium had numerous apoptotic cells. Apoptotic index of tubal epithelium was 75%. Moderate number of apoptotic cells we saw in tubal *lamina propria*, blood vessels, *tunica muscularis* and mesothelium. Cytotrophoblast, syncytiotrophoblast, extraembryonal mesenchyma and peripheral trophoblast also contained moderate number of apoptotic cells.

Antimicrobial defence proteins in tubal pregnancy: moderate number of human defensin beta 2 positive cell was found in tubal epithelium. Human defensin beta 2 containing granules were located in cytoplasm and on apical surface of plasmatic membrane. Moderate number of human defensin beta 2 positive concentrated around blood vessels. Mesothelim demonstrated numerous human defensin beta 2 positive cells. Cytotrophoblast and syncytiotrophoblast had some human defensin beta **2** positive cells. Numerous human defensin beta 2 positive cells were abundant in peripheral trophoblast.

Appearance of growth factors, their receptors, human defensin beta 2 and apoptosis in intrauterine pregnancy tissue

Transforming growth factor beta 2: moderate number of TGF beta 1 positive cells located in endometrial epithelium and connective tissue. TGF beta 1 positive macrophages and neutrophils surrounded blood vessels. Peripheral trophoblast contained numerous TGF beta 1 positive cells.

**Basic fibroblast growth factor and it`s receptor:** we found numerous FGFR1 positive structures in endometrial epithelium and smooth muscle cells. Blood vessel`s wall and nerve fibers contained moderate number of FGFR1 positive structures. Some connective tissue macrophages and neutrophils contained FGFR1.

Moderate number of bFGF positive structures was discovered in endometrial epithelium and *lamina propria*. Extraembryonal mesenchyma contained numerous, but cytotrophoblast and syncytiotrophoblast moderate number of FGFR1 positive structures. bFGF in these tissues was absent. Peripheral trophoblast showed moderate number of bFGF and FGFR1 positive cells.

Basic nerve growth factor and it`s receptor p 75: *decidua basalis* epithelium contained numerous NGF positive cells. *Decidua compacta*

accommodated numerous NGF positive cell including macrophages and neutrophils. Moderate number of nerve fibers, but few blood vessels wall`s cells expressed NGF on their *plasmolemma*. Peripheral trophoblast demonstrated numerous, but syncytiotrophoblast moderate number of NGF positive cells. Few NGF positive cells were found in extraembryonal mesenchyma. NGF positive structures were absent in cytotrophoblast.

Insulin like growth factor 1 and it`s receptor: endometrial epithelium demonstrated numerous IGF-1 and IGF-1R positive structures. Both IGF-1 and IGF-1R positive structures were absent on smooth muscle cells, endotheliocytes and nerve fibers *plasmolemma*. Moderate number of peripheral trophoblast, cytotrophoblast and syncytiotrophoblast cells expressed IGF-1. Numerous IGF-1R positive cells were abundant in peripheral trophoblast, but cytotrophoblast and syncytiotrophoblast showed moderate number of IGF-1 positive structures. Few IGF-1 and IGF-1R positive cells located in extraembryonal mesenchyma. Endometrial *lamina propria* contained moderate number of IGF-1R and few IGF-1 positive cells.

Hepatocyte growth factor: moderate number of HGF positive cells was found in endometrial epithelium. HGF positive structures were absent in nerve fibers, endotheliocytes and myocytes. Endometrial *lamina propria* contained numerous HGF positive structures. Extraembryonal mesenchyma, syncytiotrophoblast, cytotrophoblast and peripheral trophoblast accommodated numerous HGF positive cells.

Endometrial innervation: protein gene product 9.5: few PGP 9.5 positive cells were discovered in endometrial epithelium. Endotheliocytes and myocytes were negative for PGP 9.5. Cytotrophoblast, syncytiotrophoblast and extraembryonal mesenchyma were also free of PGP 9.5 positive structures. *Decidua basalis* demonstrated numerous PGP 9.5 positive nerve fibres. Peripheral trophoblast and endometrial *lamina propria* showed moderate number of PGP 9.5 positive cells.

Programmed cell death in uterine pregnancy: numerous apoptotic cells were found in gravid endometrium. Apoptotic index of endometrial epithelium was 74%. Numerous apoptotic myocytes and endotheliocytes accommodated in *decidua basalis.* Numerous peripheral trophoblast and chorionic villi cells demonstrated apoptosis.

Antimicrobial defence proteins in uterine pregnancy: numerous human beta defensin 2 positive structures were abundant in endometrial epithelium. Numerous connective tissue cells including macrophages and neutrophils were human beta defensin 2 positive. These cells concentrated around blood vessels. Cytotrophoblast, syncytiotrophoblast and peripheral trophoblast contained moderate number of human beta defensin 2 positive structures as well as extraembryonal mesenchyma

Appearance of growth factors, their receptors, neiropeptides, CK, human defensin beta 2and apoptosis in human embryo tissue

Transforming growth factor beta 1: we detected numerous TGF beta 1 positive cells in embryonic tissue such as epithelium, kidney and lungs. TGF beta 1 granules concentrated in apical cytoplasma. Numerous TGF beta 1 positive structures were in sclerogenic mesenchyma, blood vessels. Moderate number of TGF beta 1 positive cells accommodated in muscles and on nerve fibres plasmolemma.

TGF beta 1 positive cells were abundant in myocardium and pericardium. Cartilage proliferation zone and ossification centres demonstrated moderate number, but perichondrium – numerous TGF beta 1 positive structures. Extraembryonal mesenchyma showed numerous TGF beta 1 positive cells. Few TGF beta 1 positive cells concentrated in nerve tube, spinal ganglia and meninges.

Basic fibroblast growth factor and its receptor: numerous FGFR 1 positive structures were seen in embryonic tissue such as epithelium, sclerogenic mesenchyma, perichondrium and cartilage proliferation zone. *Chorda dorsalis* demonstrated a moderate number of FGFR1 positive cells. Both FGFR1 and bFGF were absent in chondrocytes.

Pleura and pericardium, mesothelium, myocardium, spinal ganglia, muscle and nerve fibers *plasmolemmae* contained moderate number of FGFR1 positive structures. Moderate number of bFGF positive cells was found in epithelium, endothelium, cartilage proliferating zone and perichondrium. Embryonic sclerogenic mesenchyma, muscle fibers, nerve fibers, spinal ganglia, meninges and *chorda dorsalis* fragments accommodated few bFGF positive structures.

Basic nerve growth factor and it`s receptor p75: numerous or moderate number of NGF positive cells were seen in embryonic organs. Moderate number of NGF positive structures located in sclerogenic mesenchyma, perichondrium, meninges and spinal ganglia. Moderate number of muscle fibers and endotheliocytes plasmolemmae exhibited NGF. Numerous nerve fibers were NGF positive. *Chorda dorsalis* fragments, cartilage proliferation zones, chondrocytes and ossification centers were NGF negative.

Numerous NGFRp75 positive structures were abundant on nerve fibres, muscle fibres and endotheliocyte plasmatic membranes. Moderate number of NGFRp75 positive cells located in spinal ganglia and extraembryonal mesenchyma. NGFRp75 was absent in embryo epithelium and future skeleton.

Insulin like growth factor 1 and its receptor: numerous IGF-1 and IGF-1R positive structures were found in embryo respiratory and digestive system as well as in kidney and skin epithelium. Both IGF-1 and IGF-1R positive structures were seen in endothelium and muscle fibers. Numerous IGF-1 and IGF-1R positive cells concentrated in embryonic liver. Sclerogenic

mesenchyma, perichondrium and ossification centers demonstrated moderate number of IGF-1 and IGF-1R positive cells.

Hepatocyte growth factor: numerous HGF positive cells were discovered in embryonic epithelia, mesenchyma and nerve system. Embryos contained numerous HGF positive endotheliocytes, muscle fibers and nerve fibers. Perichondrium and embryonic internal organ capsules demonstrated numerous HGF positive cells.

Embryonic skin, digestive, respiratory and kidney epithelium as well as liver contained a moderate number of HGF positive cells. The same quantity of HGF positive cells were found in extraembryonal mesenchyma, spinal ganglia and meninges. HGF positive cells were absent in future skeleton structures.

Innervation and CNS development:

Synaptophysin: moderate number of synaptophysin containing cells was in embryonal spinal ganglia and in connective tissue around blood vessels. Nerve fibers and muscle fibers expressed synaptophysin on their plasmatic membrane. Despite these structures synaptophysin was absent in other embryonic tissue.

Chromogranin A and B: we discovered a moderate number of chromogranin A and B positive cells only in spinal ganglia. Chromogranin A and B containing granules concentrated spinal gangliocytes cytoplasm.

Glial fibrillary acidic protein: numerous GFAP containing nerve fibers were seen in embryonic tissue. GFAP positive cells localized in spinal ganglia and in nerve tube ependimal and mantle layer. Motor neurons also were GFAP positive. Blood vessels wall contained moderate number of GFAP positive cells. Embryonic epithelium, connective tissue, muscles and skeleton elements were GFAP negative.

Protein gene product 9.5: numerous PGP 9.5 positive nerve fibers were abundant in embryonic tissue. Meninges and spinal ganglia contained a

moderate number of PGP 9.5 positive structures. Few cells in embryo liver, kidney and lungs expressed PGP 9.5.

Cytokeratin 5 and 13 in embryo tissue: all layers of embryonic skin epithelia demonstrated numerous CK 5 and CK 13 positive cells. Blood vessels wall contained few CK 5 and CK 13 positive. Embryo ossification centers contained some CK 5 and CK 13 positive cells.

Mesenchyma inducting genes in embryonic tissue: embryonic epithelium, endothelium, muscle and nerve fibers demonstrated both Msx2 and BARX1 gene product containing structures.

BARX1 were found in nerve tube motor neurons and in pseudounipolar ganglicytes of spinal ganglion. Serous membrane such as pleura and pericardium showed a moderate number of BARX1 positive cells. Numerous BARX1 positive cells were abundant in myocardium. BARX1 gene product numerously located in sclerogenic mesenchyma, perichondrium and in cartilage proliferating zone. Numerous Msx2 gene product containing chondrocyte were found. Cartilage proliferating zone accommodated numerous, but perichondrium contained few Msx2 containing cells. Both Msx2 and BARX1 positive cells localized moderately in extraembryonal mesenchyma.

Programmed cell death in embryo tissue: apoptotic cells were found in all embryonic tissue. Apoptotic index in skin, kidney, digestive and respiratory epithelium was 78%. Moderate apoptotic cells number was discovered in embryonic liver and in internal organ differentiating mesenchyma, meninges, perichondrium. Ossification centres. Extraembryonal mesenchyma demonstrated a moderate number of apoptotic cells.

Moderate number of caspase 6 positive cells demonstrated embryonic skin, lungs, kidney and digestive system. Sclerogenic mesenchyma as well as embryonic internal organ connective tissue and perichondrium contained numerous or moderate number of caspase 6 positive cells.

Cartilage proliferating zones and hyaline cartilage were free of apoptotic cells.

Antimicrobial defence proteins in embryo tissue: moderate number of human defensin beta 2 positive cells was observed in embryonic skin epithelium as well as respiratory, digestive and kidney epithelium. Liver contained moderate number of human defensin beta 2 positive cells. These cells concentrated around blood vessels. The same quantity of human defensin beta 2 positive cells was found in sclerogen mesenchyma, perichondrium and extraembryonal mesenchyma. Few human defensin beta 2 positive cells were discovered in internal organ stroma.

Data statistical analysis

A Mann-Whitney U test was used as appropriate for evaluation of significant differences. A p value <0.05 was considered to be statistically significant.

Statistically significant difference was established between bFGF and FGFR1, IGF-1 and IGF-1R, NGF and NGFRp75 and TGF beta 1 appearance in tubal and uterine tissue.

FGFR1 dominated bFGF in both tubal (p=0,008) and uterine (p=0,011) pregnancy tissue.

IGF-1R was particularly absent in tissue in a case of tubal pregnancy in comparison with uterine pregnancy (p=0,026). **IGF-1R statistically feasibly appeared more in endometrial tissue in comparison with IGF-1R appearance in tubal tissue in any case of tubal pregnancy (p=0,017).**

TGF beta 1 was absent in tubal tissue, but in endometrial tissue this factor was abundant (p=0,049).

NGF dominated NGFRp75 in both tubal (p=0,001) and uterine (p=0,011) pregnancy cases.

Analysis by Spearman's Rank Correlation Coefficient discovered medium or small positive and negative factors and correlation of their receptors.

We have detected a **small negative correlation** between FGFR1 and NGF in epithelium (rho= −0,23), FGFR1 in epithelium and PGP 9.5 in endothelium (rho= −0,15), as well as FGFR1 and bFGF in connective tissue cells (rho= −0,26), FGFR1 and TGF beta 1 in peripheral trophoblast (rho= −0,23) in a case of uterine pregnancy.

Endometrial tissue demonstrated a **small positive correlation** between FGFR1 and NGF in connective tissue cells (rho=0,21), as well as FGFR1 and NGF in nerve fibers (rho=0,39).

Medium negative correlation was found in endometrial tissue between FGFR1 and bFGF in epithelium (rho= -0,56), FGFR1 and HGF in epithelium (rho= -0,37), FGFR1 and HGF in connective tissue cells (rho= −0,42), FGFR1 and PGP 9.5 in nerve fibers (rho= −0,4).

We identified **a small negative correlation** between FGFR1 and NGF in epithelium (rho= −0,22), FGFR1 and bFGF in connective tissue cells (rho= −0,21) in tubal tissue in a case of tubal pregnancy.

Tubal tissue demonstrated **small positive correlation** between FGFR1 and bFGF in muscle fibers (rho=0,19), FGFR1 and IGF-1 in peripheral trophoblast (rho=0,16), FGFR1 and PGP 9.5 in peripheral trophoblast (rho=0,22), FGFR1 and IGF-1 in connective tissue cells (rho=0,21) in a case of ectopic pregnancy.

Small negative correlation was detected between FGFR1 and HGF in epithelium (rho= −0,58), FGFR1 and HGF in nerve fibers (rho= −0,48), FGFR1 and PGP 9.5 in nerve fibers (rho= -0,425) in tubal tissue.

Medium positive correlation between FGFR1 and NGF in peripheral trophoblast (rho=0,49) was established in a case of tubal pregnancy.

The Kruskal Wallis test was used for comparison of a number of samples testifying statistically significant difference between implantation site (tube or endometrium) and

- bFGF in epithelium ($p< 0,0001$)
- IGF-1R in epithelium ($p<0,0001$)
- IGF-1R in cytotrophoblast ($p <0,0001$)
- IGF-1R in connective tissue cells ($p<0,0001$)
- IGF-1 in peripheral trophoblast ($p<0,0001$)
- TGF beta 1 in peripheral trophoblast ($p<0,0001$)
- HGF in extraembryonal mesenchyma ($p<0,0001$)
- HGF in connective tissue cells ($p<0,0001$)
- NGF in epithelium ($p<0,0001$)
- NGF in muscle fibres ($p<0,0001$)
- NGF in endothelium ($p<0,0001$)
- NGF in connective tissue cells ($p<0,0001$)
- NGF in nerve fibres ($p<0,0001$)
- Human defensin beta 2 in cytotrophoblast ($p<0,0001$)
- Human defensin beta 2 in syncytiotrophoblast ($p<0,0001$)
- Human defensin beta 2 in peripheral trophoblast ($p<0,0001$)
- Human defensin beta 2 in connective tissue cells ($p<0,0001$)
- PGP 9.5 in epithelium ($p<0,0001$)
- PGP 9.5 in peripheral trophoblast ($p<0,0001$)

DISCUSSION

Population of the research and risk factors

We included two patients' groups in our descriptive study:

1. patients with unwanted physiologic uterine pregnancy,
2. patients with ectopic tubal pregnancy.

Ectopic pregnancy usually occurs in 30 years old women (Kamwendo et al., 2000). The average age of patients from tubal pregnancy group was 29,7 years, but in uterine pregnancy group the average age was 30,8 years (p=0,62). Gestation time was not significantly different in both groups: 6,5 weeks since last menstrual period in uterine pregnancy group and 6,7 weeks in tubal pregnancy group (p=0,21).

PID is known to be one of ectopic pregnancy risk factors (Varma and Gupta, 2009). The average number of PID episodes in tubal pregnancy group was 0,7 (n=10) and in uterine pregnancy group 0,3 (n=3), which was not statistically significant difference (p=0,062). We can explain this fact with a small number of patients involved in our study.

PID incidence positively correlates with the incidence of STD, especially with the incidence of *Chlamydia trachomatis* and *Nesseria gonorrhoea* infections (Haggerty et al., 2010). Only one patient from both groups had a documented STD (Chlamydia infection). It has been established, that the incidence of *Chlamydia trachomatis* infection in European Union varies from 1,7 to 17% (Fenton et al., 2004).

We have documented patients' partners, because STD incidence is dependent on sexual behaviour. PID risk increases, if a woman has had more than one sexual partner within last 30 days (Jossen at al., 1996), or if she has had more than 4 partners within her life (Wolner-Hanssen et al., 1990). The

average partner count in uterine pregnancy group was 2,66 partners, but in tubal pregnancy group 2,75 (p=0,9).

Patients from both groups were not using any contraception method. Only one patient from tubal pregnancy group has had IUD for last two years. IUD decreases blastocyst implantation rate for 99% (Kaneshiro et al., 2010), but do not influence ovulation, fertilization and ectopic implantation. Xiong proved that IUD is neither tubal pregnancy nor ruptured tubal pregnancy risk factor (Xiong et al., 1995).

Each patient from uterine pregnancy group had more than one pregnancy before termination of current pregnancy (4,2 on average), but for two patients from tubal pregnancy group it was their first pregnancy (2,4 on average). Uterine pregnancy patient terminated previous pregnancies more commonly (2,4 abortions on average), than patients from tubal pregnancy group (0,94 abortions on average p=0,004).

Smoking was shown to be one of ectopic pregnancy risk factors (Farquar, 2005), but there were more smoking women in uterine pregnancy group (n=7) than in tubal pregnancy group (n-=3; p=0,013) in our study. We can explain this fact with lower social and economic status of the patient which chooses unwanted pregnancy termination (Pedersen et al., 2006) and a small patient count involved in our study.

It was not the object of our study to do the epidemiologic analysis of tubal pregnancy. We have concluded, than despite pregnancy and abortion count, there were no differences in anamnesis data between the groups. This fact is important for us because it is crucial to detect possible differences and associated pathologies, which could impact morphologic scene in embryo, endometrial and tubal slides.

Growth factors and their receptor
in uterine and tubal implantation tissue

TGF beta 1 is important for function of reproductive system (Godkin et al., 1998; Jones et al., 2006). This factor participates in endometrial remodelling (Stoikos et al., 2008) and trophoblast invasion (Lyall et al., 2001; Simpson et al., 2002). TGF beta 1 appears on both *conceptus* and endometrial surfaces in periimplantation period (Gupta et al., 1996; 1998a; 1998b), regulates endometrial transformation, *conceptus* adhesion (Massuto et al., 2010) and trophoblast invasion in endometrium (Geisert et al., 1982; Bazer et al., 1987).

We established abundant TGF beta 1 in endometrial epithelium, some peripheral trophoblast cells, macrophages and neutrophils contained TGF beta 1. This finding testifies TGF beta 1 importance during endometrial transformation in implantation site.

Zhao has found all TGF beta 1 isoforms in tube and concluded, that TGF beta 1 participates in tube physiology (Zhao et al., 1994). TGF beta 1 is shown to modulate tubal microenvironment and to be autocrine or paracrine factor regulating embryonic development during periimplantation (Arganaraz et al., 2010). Patients with tubal infertility have increased TGF beta 1 immunexpression in adhesion sites, so TGF beta 1 forces adhesions in tube (Sun et al., 2009). The same authors' group later established a negative correlation between TGF beta 1 expression and pregnancy possibility in patients after surgical adhesions correction independently from severity of adhesions and patient`s age (Li et al., 2011).

There were none of TGF beta 1 positive structures in tubal tissue involved in our study. Taking into account all decrypted literature data on TGF beta 1 we can conclude, that ectopic implantation in our patients is not due to tubal adhesions. Absence of TGF beta 1 in tubal tissue testifies disrupted

molecular interactions between *conceptus* and mother tissue continued with impossibility to develop ectopic pregnancy.

bFGF expresses in endometrial tissue during the menstrual cycle (Rusnati et al., 1990). There is an increased estrogens dependent bFGF expression during pregnancy (Wordinger et al., 1994; Samathanam et al., 1998). Both bFGF receptor expressions are found in endometrium – FGFR1 and FGFR2 (Welter et al., 2004). Edwards detected bFGF and its receptors to be found in endometrial luminal, crypt epithelium and in stroma during implantation. He showed increased FGFR1 and FGFR2 expression in a case of VEGF loss, so bFGF can force angiogenesis for successful implantation and placentation (Edwards et al., 2011).

We saw numerous FGFR1 positive structures in all tubal wall layers. The same FGFR1 appearance was established in endometrial tissue. Some bFGF positive cells were found in endometrial epithelium and decidual connective tissue. Embryonic tissue such as syncytiotrophoblast, cytotrophoblast and extraembryonal mesenchyma demonstrated numerous FGFR1 positive structures, but bFGF appeared less common.

We conclude that bFGF and FGFR1 appears in equal quantity in implantation site and in trophoblast cells during tubal and uterine pregnancy. Thus this factor participates in angiogenesis and tissue remodelling. We detected FGFR1 dominated bFGF in physiologic and pathologic implantation site in our study. Probably, FGFR1 was more abundant, because of blastocyst induced tissue compensatory remodelling in tubal and endometrial tissue.

NGF participates in different reproductive processes: it regulates mast cell degranulation in implantation site (Bose at al., 2009), installs FSH receptor expression and estrogens synthesis, by regulation of ovarian folliculocytes sensitivity to gonadotrophins (Salas et al., 2006). Progesterone keeps local NGF concentration in uterus, which is necessary for pregnancy progress (Shi et al., 2006).

We discovered moderate number of NGF positives structures in tubal tissue: epithelium, smooth muscle cells and in nerve fibres in a case of tubal pregnancy. The same picture was seen in a case of blastocyst implantation in to endometrium: numerous NGF positive cells were in *decidua basalis* epithelium, some peripheral trophoblast and cell in compact layer including macrophages and neutrophils were NGF positive.

Tubal *lamina propria* and *tunica muscularis* as well as *decidua basalis* stroma contained NGFRp75 positive nerve fibres only. There were none of statistically significant differences in NGF and NGFRp75 appearance between tubal and endometrial tissue.

PGP 9.5 which is known to be nerve and neuroendocrine cells marker was found in tubal epithelium and nerve fibres. There were PGP 9.5 positive neuroepithelial bodies found in tubal epithelium. Some peripheral trophoblast and tubal *lamina propria* cells expressed PGP 9.5. *Decidua basalis* epithelium, nerve fibres, peripheral trophoblast and connective tissue cells demonstrated PGP 9.5. So, immunohistochemical appearance of PGP 9.5 in tubal and endometrial tissue was equal.

There were no NGF, NGFRp75 and PGP 9.5 alterations in tubal wall tissue. We conclude, that NGF, NGFRp75 and PGP 9.5 are important factors for implantation and these factors are not involved in tubal implantation.

Endometrial decidual cells express IGF-1 in implantation site (Kapur et al., 1992). This is absolutely necessary for embryo implantation. We detected moderate number of IGF-1 and numerous IGF-1R positive cells in endometrial epithelium. There was no statistically significant difference between IGF-1 and IGF-1R appearance in decidual tissue, so both IGF-1 and IGF-1R participated in endometrial remodelling, embryo implantation, trophoblast invasion and promotes successful pregnancy development.

IGF-1 was abundant in tubal epithelium in our study, but only some epitheliocytes contained IGF-1R in their apical cytoplasm. The lack of IGF-1R

in tubal pregnancy tissue testifies cells proliferation and trophoblast invasion alterations in tissues, where pathologic implantation takes place. Embryo development basic processes, such as angiogenesis, trophoblast invasion and cell growth are disrupted, because of IGF-1R lack. It is possible, that lack of IGF-1R in implantation site could be compensatory adaptation mechanism limiting pathologic process.

HGF participates in cyclic remodelling of endometrium, regulating oestrogens and progesterone dependent cell proliferation in mice (Zhang, 2010). Oestrogens regulate HGF synthesis by endometrial fibroblasts in humans. HGF producing fibroblasts could be involved in endometriosis and endometrial cancer (Coleman et al., 2009; Felix et al., 2010). HGF is widely found in human reproductive system fluids including oviduct (Srivastava et al., 1999). HGF regulates trophoblast migration (Rajaraman et al., 2010) and invasion (Kauma et al., 1999) during implantation.

HGF appeared in equal quantity in both tubal and endometrial tissue in our research. So, we conclude, that HGF is not involved in pathologic implantation.

MMP 2 is one of the most important human trophoblast invasion regulators in human tissue during early pregnancy. Seval considers MMP 2 to be the first mediator, which promotes trophoblast invasion into decidual endometrium as well as remodelling and angiogenesis (Seval et al., 2004). Trophoblast cells express MMP 9 *in vitro* and *in vivo* (Peters et al., 1999). MMP 9 activation is necessary for extracellular matrix proteolysis during blastocyst invasion process (Martinez-Hernandez et al., 2011).

We found MMP 2 and MMP 9 to be widely found in tubal wall and chorionic willi tissue. We conclude that MMP 2 and MMP 9 dependent trophoblast invasion is not impaired, so MMP 2 and MMP 9 dysfunction is not involved in ectopic implantation,

Programmed cell death in implantation site

Apoptosis provides cyclic changes in human endometrium. This process is progesterone and oestrogens dependent and widens to lutein phase (Otsuki, 2001; Sengupta et al., 2003).

Tubal apoptotic cells count also changes after ovulation towards tubouteral junction, so apoptosis is involved in oocyte maturation and fertilization (Urhausen et al., 2011). Assisi detected tubal apoptotic activity to be increased after ovulation, which provides dynamic changes in tube and tissue remodelling through reproductive cycle (Assisi et al., 2011).

We discovered total apoptosis in gravid endometrium and chorionic villi using TUNEL method. Tubal epithelium and connective tissue demonstrated a widely distributed apoptosis. We consider this finding to be connected to tissue remodelling due to blastocyst nidation. There were no statistically significant differences in apoptotic cell distribution between tubal and uterine tissue.

Antimicrobial defence proteins in implantation site

Antimicrobial defence proteins were analyzed by identifying human defensin beta 2 in endometrial and tubal tissue. Neutrophils secrete human alpha and beta defensins in endometrial stroma and do not influence embryo implantation in uterus (Das et al., 2007). Tubal epitheliocytes contain human defensin beta 2 in apical cytoplasm in normal oviduct and adhesions changed tube (Hu et al., 2010).

Human defensin beta 2 containing cells were widely seen in tubal and endometrial tissue. Difference in human defensin beta 2 positive cells distribution between tubal and endometrial tissue was not statistically

significant. Human defensin beta 2 participates in both processes of physiologic and tubal implantations.

Growth factors and their receptors in embryonic tissue

TGF beta 1 controls cell proliferation, migration, apoptosis and differentiation during embryogenesis (Yang et al., 2003). This factor provides epithelial – mesenchymal interaction in prenatal and postnatal development (Zavadil and Boettinger, 2005), as well as determines future skeleton shape (Hall and Miyake, 2001). TGF beta 1 is widely appeared in embryonic tissue from 4 to 6 embryonal development weeks (Kukanova and Pilmane, 2007).

Embryonal tissue demonstrated abundant TGF beta 1 immunreactivity in our research. It is established that epitheliocytes express mesenchymal markers and *vice versa* due to TGF beta 1 control of cell differentiation (Chai et al., 2010). TGF beta 1 rich distribution in embryonic tissue testifies this factor' active involvement in the first trimester organogenesis.

One more mesenchymal origin cell marker bFGF (Chai et al., 2010) was richly found in embryonal tissue. FGF family activity provides mesenchymal differentiation, migration and positively or negatively regulates important blastocyst gene expression (Hardy et al., 2011).

We discovered numerous FGFR1 and moderate number of bFGF positive cells in embryonic tissue involved in our study. We suggest bFGF to regulate endodermal, ectodermal and mesodermal origin cell differentiation during embryogenesis. bFGF and FGFR1 are one of the most widely found factors in human embryogenesis.

Despite cartilage proliferation zone, ossification centres and chondrocytes, numerous or moderate number of NGF positive structures were found in all embryonic tissue. NGFRp75 was abundant on nerve fibres, muscle

fibres and endotheliocyte plasmatic membranes. NGF provides human embryo cell survival and is necessary for blastocyst implantation, further development and pregnancy progress resulting in live birth (Tometten et al., 2005; Toti et al., 2006).

Research on IGF-1 and its receptors is of interest in modern reproductive physiology field nowadays. Mother IGF-1 stimulates fetal growth by nutrition molecules transplacentar transport activation (Iwashita, 1994). Compensatory increasing of maternal IGF-1 in placental tissue is established in a case of fetal intrauterine growth retardation (Holmes et al., 1999; Ozkan et al., 2008a). Kamei established alterations in IGF-1 interaction with its receptor in hypoxic zebra fish embryos: decreases IGF-1R expression and increases synthesis of insulin like a binding protein (Kamei et al., 2011).

Research on genes shows IGF-1 importance for embryo development. Survival of IGF-1, IGF-2 and IGF-1R knockout mice (Liu et al., 1993) and human embryos with nature IGF-1, IGF-2 and IGF-1R mutations are not possible because of severe developmental malformations (Klammt et al., 2008).

We found IGF-1 and IGF-1R positive cells in all human embryo tissues. Brogilo considers IGF-1 impact on target cells to be crucial for embryo growth and development (Brogiolo et al., 2001). This factor and receptor interaction is believed to be an evolutionary regulatory system, which starts early in embryogenesis and determines embryo survival. Our data on IGF-1 and IGF-1R distribution in embryonic tissue from 5 to 12 embryonal development weeks testifies the significance of these factor and receptor in embryogenesis. These findings are confirmed by other authors' data (Schlueter et al., 2007; Le Rotin, 2008).

Mesenchymocytes express HGF, which is a morphogenic factor participating in organogenesis during embryogenesis (Barros et al., 1995; Ohmichi et al., 1998). Mesodermal HGF expression regulates cell migration to

dermatomyosclerotome and determines the shape of future skeleton (Kawashima-Ohya et al., 2011).

HGF was widely distributed in embryonic epithelium, mesenchyma and nerve system in our study. This fact confirms HGF significance in further embryonal development of 5/6 gestation weeks` embryos.

Innervation and central nerve system development

Synaptophysin participates in calcium transport, neurotransmission, growth of nerve cells. This neuropeptide is crucial for CNS and PNS synaptogenenesis in early embryogenesis (Wiedenmann and Franke, 1985; Buffa et al., 1986; Gould et al., 1986). We found numerous synaptophysin positive cells in embryonic nerve system, spinal ganglia and in some blood vessels surrounding connective tissue cells.

Chomogranins controls secretory granules biogenesis and are found in all neuroendocrine cells (Larsson et al., 1992; Tischler, 2002; Hendy et al., 2006). These prohormones are expressed in tissues rich in neuroendocrine cells, such as adrenal glands, digestive system and pancreas during embryogenesis (Kent and Coupland, 1989; Mahata et al., 1993; Totzauer et. al., 1995; Kameda et al., 1998).

Chromogranins A and B containing granules were found in cytoplasma of spinal ganglion ganglionar cells of 5-12 gestation weeks embryos involved in our study. We suggest this finding proves synthesis of other neuropeptides (synaptophysin) by gangliocytes.

GFAP regulates neuronal glial cells differentiation and migration during embryogenesis, but in postnatal life becomes an astrocyte marker. Neurones, neuronal glial cells and ependimal glial cells develop from one

GFAP expressing progenitor cell (Morest and Silver, 2003; McDermott et al., 2005).

We found GFAP positive cells to be localized in spinal ganglia, nerve tube ependimal and mantle layer and in motor neurones. These nerve tube GFAP positive cells considered GFAP to be involved in nerve cell differentiation, migration and regionalization.

Cytokeratin 5 and 13 in embryonic tissue

Both CK 5 and CK 14 are found in basal layer of stratified epithelium (Lersch and Fuchs, 1988; Casatorres et al., 1994). These cells are above layers` progenitor cells – undifferentiated keratinocytes (Byrne et al., 1994; Fuchs, 2007).

CK 13 is unkeratinized epithelium marker (Gao and Mackenzie, 1992), but this fact does not limit CK 13 functions. Olson established CK 13 expression by human luminal endometrial epitheliocytes during secretory phase and by rabbit luminal endometrial epitheliocytes during periimplantation (Olson et al., 2002).

Embryonic stratified epithelium expressed CK 5 and CK 13 in our study. Possibly, CK 5 and CK 13 could be the markers for undifferentiated keratiocytes and for stratified epitheliocytes, or CK 5 and CK 13 expression could be find in all undifferentiated epitheliocytes. So, traditional opinion on CK tissue expression cannot be confirmed.

Mesenchyma inducting genes in embryo tissue

BARX1 gene regulates the development of craniofacial ectomesenchyma, bone and stomach (Tissier-Seta et al., 1995; Tucker et al., 1998).

BARX1 gene product was widely distributed in embryonic tissue involved in our study.

Msx2 is a gene coding DNA binding protein, which modulates transcriptional activity (Takahashi et al., 1996) and promotes cell growth (Davidson, 1995). We detected Msx2 gene product in different organs of mesodermal origin: in lungs, heart, sclerogenic mesenchyma, muscle fibres, blood vessel wall and serous membranes. Embryonic oral cavity, tongue and salivary glands` ectodermal origin epithelium demonstrated Msx2 positive cells. This fact could be explained by Msx2 provided adjacent mesenchyma induction, which is necessary for ectodermal derivates' development. Msx2 is known to be the epithelial – mesenchymal interactions regulator and find transcription and gene product in salivary glands (Jaskoll et al., 1998).

BARX1 and Msx2 gene products are transcriptional factors having integrative role in embryonic tissue. These factors provide the development of mesenchymal origin organs and differentiation of ectodermal cells, providing molecular interaction between germ layers (Kukanova and Pilmane, 2007).

Programmed cell death in embryo tissue

Apoptosis provides the regulation of cell proliferation and differentiation during embryogenesis. TUNEL is an approved method to detect apoptotic cells in human blastocyst and embryo (Gavreli et al., 1992; Byrne et al., 1999) We discovered widely appeared apoptotic cells in human embryo tissue from 5 to 12 embryonal development weeks.

We cannot approve such a wide apoptosis to be found *in vivo.* Probably this apoptotic cell distribution was due to the influence of pregnancy termination manipulation on embryonic cells. We believe that apoptosis is a physiologic process during embryogenesis and goes in parallel to cell

differentiation, limiting distribution of undifferentiated cells in tissue to genetically determined quantity.

The distribution of one of programmed cell death agents – caspase 6 was similar to TUNEL positive cells distribution. We conclude, that programmed cell death takes place in human embryo development during implantation and organogenesis. Programmed cell death is realised by caspase 6 activation.

Antimicrobial defence proteins in embryo tissue

Antimicrobial defence proteins protect both mother and fetus. This protection is realized by human defensins – group of antimicrobial defence proteins. Vento described defensins to be found in bronchial fluid of neonates delivered before the term and concluded innate antimicrobial defence to act before the fetus becomes maturated for postnatal life (Vento et al., 2010).

Our study showed a wide distribution of human defensin beta 2 in human embryo tissue. We suggest that innate antimicrobial defence starts to act early and provides antimicrobial defence of 5/6 gestation weeks embryos.

At the end of discussion part we have to accept that the analysis of 5/6 to 12 gestational weeks' embryos, which further development was terminated forcibly, it is not possible to explain physiologic processes *in vivo*. We need to involve more embryos of different gestation ages and to compare our findings with fetal period for complete understanding of growth factors and genes participating in human prenatal development. Such a research on normally developed human embryos in physiologic conditions *in vitro* and *in vivo* is not possible because of ethical considerations

CONCLUSIONS

1. There is the deficit on IGF-1R in the blastocyst implantation site in a case of tubal pregnancy. The deficit of IGF-1R in fallopian tube might be a result compensatory adaptation mechanism lead to control the pathologic process.
2. In both spontaneous and tubal blastocyst implantation sites FGFR1 is more abundant than bFGF. Probably this is due to pregnancy caused tissue remodelling.
3. FGFR1, bFGF, NGF, NGFRp75 PGP 9.5, HGF, human defensin beta 2, MMP 9 and MMP 2 are widely appeared in oviduct tissue in a case of tubal pregnancy and take part in tubal pregnancy morphopathogenesis.
4. TGF beta 1, IGF-1, IGF-1R, bFGF, FGFR1, NGF, NGFRp75, HGF, PGP 9.5 are widely found in endometrial tissue in a case of uterine pregnancy and take part in blastocyst implantation.
5. TGF beta 1, IGF-1, IGF-1R, bFGF, FGFR1, NGF, NGFRp75, HGF, PGP 9.5 are widespread in human embryo tissue from 5/6 to 12 embryonic development week and take part in embryogenesis.
6. Apoptosis is a widespread process, distributed in tubal, uterine blastocyst implantation site and 5/6-12 gestation weeks old embryo tissue, involved in tissue remodelling and differentiation.
7. Human defensin beta 2 innate response starts at 5/6-12 gestation weeks.
8. BarX1 and Msx2 gene products are widely distributed in human embryonic tissue, probably providing mesenchymal organ development and regulating ectodermic origin cells differentiation due to epithelial – mesenchymal induction in 5/6-12 gestation weeks.
9. GFAP is a nerve tissue progenitor cells both glial and neurons marker, synaptophysin provides CNS cell contacts forming, but chromogranin

A and B are involved in neiropeptide (synaptophysin) synthesis 5/6-12 gestation weeks.

10. Human embryo stratified epithelia express CK 5 and CK 13, that testify early fenotyping of epithelial tissue.

SCIENTIFIC PUBLICATIONS

Published papers

1. *Kukanova A., Pilmane M.* Distribution of Neuropeptides in Human Embryonic Central Nervous System. RSU zinātniskie raksti, 2006; 302 – 304
2. *Kukanova A., Pilmane M.* Distribution of Some Growth Factors and Appearance of Some Genes in Different Tissues of Human Embryo. RSU Collection of Scientific Papers, 2007; 21 – 24
3. *Kukanova A., Pilmane M., Rezeberga D.* Occurrence of growth factors and their receptors in tubal pregnancy – affected tissue. Proceedings of the Latvian Academy of Sciences, 2010; 64 (4): 20 – 30
4. *Miskova A., Pilmane M., Rezeberga D.* Augšanas faktoru un antimikrobās olbaltumvielu sadalījums dzemdes un olvada grūtniecības gadījumā. RSU zinātnisko rakstu krājums, 2010; 183–190
5. *Miskova A., Pilmane M., Rezeberga D.* Immunohistochemical distribution of IGF-1, bFGF and their receptors in decidual, embryonic and tubal human pregnancy tissue. In: Papers on Anthropology 2011, (20); 272–288

Thesis published in Latvia

1. *Kukanova A., Pilmane M., Rezeberga D* Distribution of some Growth Factors and Genes in Different Tissue if Hyman Embryo. Rīgas Stradiņa Universitātes 6. zinātniskās konferences tēzes, 29.-30. marts, 2007: 71

2. *Kukanova A., Pilmane M., Rezeberga D.* Distribution of some Growth Factors and Genes in Different Tissue if Hyman Embryo. The abstract book of Baltic Morphology V, 19-20 November, Riga, Latvia, 2007: 43
3. *Kukanova A., Pilmane M., Rezeberga D.* Augšanas faktori un apoptoze olvada grūtniecības gadījumā". Rīgas Stradiņa Universitātes 7. zinātniskās konferences tēzes, 13.-14. marts, 2008: 60
4. *Kukanova A., Pilmane M., Rezeberga D.* Augšanas faktori olvada grūtniecības gadījumā. The abstract book of Baltic International Conference in Obstetrics and Gynecology and 5th Congress of Latvian Obstetricians and Gynecologists, 10-11 October, Riga, Latvia, 2008: 17. The best poster presentation award.
5. *Kukanova A., Pilmane M., Rezeberga D.* Cilvēka defensīna beta 2. sadalījums olvadu audos ārpusdzemdes grūtniecības gadījumā. Rīgas Stradiņa Universitātes 8. zinātniskās konferences tēzes, 2.-3. aprilis, 2009: 71
6. *Kukanova A., Pilmane M., Rezeberga D.* Insulin like growth factor 1 and its receptor appearance in pregnancy associated tissue. 6. Latvijas Ārstu kongresa tēzes, 19.-21. jūnijs, 2009: 46
7. *Kukanova A., Pilmane M., Rezeberga D.* IGF-1, bFGF, TGF beta 1 and their receptors in different cases of embryo implantation. Rīgas Stradiņa Universitātes 9. zinātniskās konferences tēzes, 18.-19. marts, 2010: 77
8. *Miskova A., Pilmane M., Rezeberga D.* FGFR1 un bFGF sadalījums cilvēka embrija un grūtniecības audos. Rīgas Stradiņa Universitātes 10. zinātniskās konferences tēzes, 14.-15. aprilis, 2011: 74
9. *Miskova A., Pilmane M., Rezeberga D.* NGF and NGFRp75 appearance in embryo tubal and uterine implantation. The abstract book of 6th Congress of Latvian Obstetricians and Gynecologists and the 4th RCOG Eurovision Conference, 13.-15. October, Riga, Latvia, 2011: 85

Thesis published abroad

1 *Kukanova A., Pilmane M., Rezeberga D.* Факторы роста TGF beta 1, NGF, bFGF, IGF-1 и их рецепторы в случае трубной беременности. Проблемы репродукции, 2009; 241 – 242

2 *Kukanova A., Pilmane M., Rezeberga D.* Human defensine beta 2 distribution in pregnancy associated tissue. The abstract book of Baltic Morphology V, 27-28 August, Kaunas Lithuania, 2009: 10

3 *Kukanova A., Pilmane M., Rezeberga D.* Distribution of growth factors and their receptors in tubal pregnancy affected tissue. Journal of Perinatal Medicine, 2009; 37: 806-807

4 *Kukanova A., Pilmane M., Rezeberga D.* NGF, NGFRp75 and PGP9.5 immunoreactivity in tubal pregnancy tissue. Early Pregnancy Winter Course. Early pregnancy standarts, protocols and guidelines, 10-11 december, Rotterdam, Netherland, 2010; 239-240

5 *Kukanova A., Pilmane M., Rezeberga D.* IGF-1 appearance in embryo tubal and uterine implantation. The abstract book of 6th International Conference on the Female Reproductive Tract, 20-23 May, Frauenchiemsee, Germany 2011: 13

6 *Miskova A., Pilmane M., Rezeberga D.* FGFR1 and bFGF appearance in human embryo and pregnancy tissue. Human Reproduction, 2011; 26 (1): i214

7 *Miskova A., Pilmane M., Rezeberga D.* Immunohistochemical distribution of IGF-1, bFGF and their receptors in decidual, embryonic and tubal human pregnancy tissue. The abstract book of Baltic Morphology VI, 22-23 September Tartu, Estonia, 2011: 73

APPROBATION OF THE STUDY

1. 30.03.2007 – poster on RSU 6 scientific conference „Distribution of some Growth Factors and Genes in Different Tissue if Hyman Embryo"
2. 13.06.2007 – II place diploma on RSU XX Resident scientific clinic conference „Actualities in medicine" for oral presentation „Distribution of Neuropeptides in Human Embryonic Central Nervous System".
3. 19.–20. 11.2007. – poster on Baltic Morphology 4th Scientific conference „Distribution of some Growth Factors and Genes in Different Tissue if Hyman Embryo" Riga Latvia.
4. 13.03.2008 – poster on RSU 7 scientific conference „Augšanas faktori un apoptoze olvada grūtniecības gadījumā".
5. 11.10.2008 – The best poster award on Baltic International Conference in Obstetrics and Gynecology and 5th Congress of Latvian Obstetricians and Gynecologists for „Augšanas faktori olvada grūtniecības gadījumā" Riga, Latvia.
6. 19. –22.01.2009 – oral presentation of „TGF beta 1, NGF, bFGF, IGF-1 and their receptors in patients with tubal pregnancy" on Third International Congress on Reproductive Medicine Moscow, Russia.
7. 2.–3.04.2009 – oral presentation on RSU 8 scientific conference „Cilvēka defensīna beta 2 sadalījums olvadu audos ārpusdzemdes grūtniecības gadījumā".
8. 19.–21.06.2009 – poster on the 6th Congress of Latvian Doctors „Insulin like growth factor 1 and its receptor appearance in pregnancy associated tissue".
9. 27.–28.08.2009 augusts – oal presentation of „Human defensin beta 2 distribution in pregnancy associated tissue" on the 5th biannual Scientific Conference „Baltic Morphology 2009" Kaunas Lithuania.

10. 24.–28.10.2009 – poster "Distribution of growth factors and their receptors in tubal pregnancy affected tissue" on 9th World Congress of Perinatal Medicine Berlin, Germany.
11. 10.–11.12.2009 – oral presentation of "NGF, NGFR75 and PGP 9.5 immunoreactivity in tubal pregnancy tissue" on ESHRE Campus Symposium Early Pregnancy Winter Course Rotterdam, Netherlands.
12. 18.–19.03.2010 – oral presentation of „IGF-1, bFGF, TGF beta 1 and their receptors in different cases of embryo implantation" on RSU 9 scientific conference .
13. 09.06.2010 – II place diploma for „Augšanas faktoru un antimikrobu olbaltumvielu sadalījums dzemdes un olvada grūtniecības gadījumā" on XXII Resident scientific clinic conference.
14. 14.–15.04.2011 – oral presentation of „FGFR1 un bFGF sadalījums cilvēka embrija un grūtniecības audos" on RSU 10 scientific conference
15. 20.–23.05.2011 – poster „IGF-1 appearance in embryo tubal and uterine implantation" on 6th International Conference on the Female Reproductive Tract Frauenchemsee, Germany.
16. 03.–06.07.2011 – poster „FGFR1 and bFGF appearance in human embryo and pregnancy tissue" on The 27th Annual Meeting of EHSRE Stockholm Sweden.
17. 20.–24.09.2011 – poster „Immunohistochemical distribution of IGF-1, bFGF and their receptors in decidual, embryonic and tubal human pregnancy tissue" on Conferences Baltic Morphology VI Tartu, Estonia.
18. 14.–15.10.2011 – poster „NGF and NGFRp75 appearance in embryo tubal and uterine implantation" on 6th Congress of Latvian Obstetricians and Gynaecologists and the 4th RCOG Eurovision Conference. Riga, Latvia.

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