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EDITOR-IN-CHIEF
Hans van Bokhoven, Professor, PhD, Department of Human Genetics and Cognitive Neurosciences, Radboud university Nijmegen Medical centre, PO Box 9101, 6500 HB Nijmegen, The Netherlands

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World Journal of Medical Genetics
Room 903, Building D, Ocean International Center, No. 62 Dongsihuan Zhonglu, Chaoyang District, Beijing 100025, China
Telephone: +86-10-59080039
Fax: +86-10-85381893
E-mail: editorialoffice@wjgnet.com
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REVIEW

Genome variation in the trophoblast cell lifespan: Diploidy, polyteny, depolytenization, genome segregation

Tatiana G Zybina, Eugenia V Zybina

Tatiana G Zybina, Eugenia V Zybina, Laboratory of Cell Pathology, Institute of Cytology, Russian Academy of Sciences, 194064 St.-Petersburg, Russian Federation
Author contributions: Zybina TG and Zybina EV contributed equally in design and writing of the manuscript as well as to preparing illustrations for this paper.
Supported by The Program "Molecular and Cell Biology" of the Russian Academy of Sciences
Correspondence to: Tatiana G Zybina, ScD, Laboratory of Cell Pathology, Institute of Cytology, Russian Academy of Sciences, Tikhoretsky ave., 4, 194064 St.-Petersburg, Russian Federation. zybina@mail.cytspb.rssi.ru
Telephone: +7-812-2970341 Fax: +7-812-2970341
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Abstract

The lifespan of mammalian trophoblast cells includes polyploidization, its degree and peculiarities are, probably, accounted for the characteristics of placenta development. The main ways of genome multiplication-endoreduplication and reduced mitosis-that basically differ by the extent of repression of mitotic events, play, most probably, different roles in the functionally different trophoblast cells in a variety of mammalian species. In the rodent placenta, highly polyploid (512-2048c) trophoblast giant cells (TGC) undergoing endoreduplication serve a barrier with semiallogenic maternal tissues whereas series of reduced mitoses allow to accumulate a great number of low-ploid junctional zone and labyrinth trophoblast cells. Endoreduplication of TGC comes to the end with formation of numerous low-ploid subcellular compartments that show some signs of viable cells though mitotically inactive; it makes impossible their ectopic proliferation inside maternal tissues. In distinct from rodent trophoblast, deviation from (2^n)c in human and silver fox trophoblast suggests a possibility of aneuploidy and other chromosome changes (aberrations, *etc.*). It suggests that in mammalian species with lengthy period of pregnancy, polyploidy is accompanied by more diverse genome changes that may be useful to select a more specific response to stressful factors that may appear occasionally during months of intrauterine development.

Key words: Cell cycle; Endocycle; Polyploidy; Genome; Aneuploidy; Trophoblast; Placenta

Core tip: In rodent placenta, differentiation of secondary trophoblast giant cells give an example of the irreversible endoreduplication (up to 1024c and higher) that, however, results in formation of low-ploid subcellular compartments uncapable of mitotic proliferation. In the mammalian species with lengthy period of pregnancy, more diverse genome changes may be useful to select a more specific response to stressful factors that may appear occasionally during months of intrauterine development.

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INTRODUCTION

Genome multiplication in the cells of placental trophoblast is a unique phenomenon among the mammalian and other vertebrate tissues. By now, ontogenesis of rodent trophoblast cells is fairly characterized and serves as a model for studying normal and pathological development of placenta. The main peculiarities of the trophoblast cell lifespan in the rodent placenta is a genome reproduction due to cell cycle reduction up to two phases-S and

G, the multifold repeat of which results in high level of cell polyploidization-up to 512-2048 and higher[1-4] that involves polytenization[5]. Beginning from the second half of pregnancy, a significant part of the secondary giant trophoblast cells undergo depolytenization and genome segregation with subsequent isolation of numerous small nuclear fragments detaching from the giant nucleus[5-8].

A range of trophoblast cell populations do not leave mitotic cycle (a part of them probably represent the trophoblast stem cells); the cells and their derivatives form cell population of lower ploidy levels (2c-32c) at the account of uncompleted mitoses[5-7,9,10]. By now, a noticeable data are available in the polyploidy in the other mammalian groups, their modes of polyploidization being different from the rodent ones[11,12]. Simultaneously, the more and more data appear on the role of polyploidy in formation of different mammalian tissues[13-19]. In particular, in recent publications a great attention is drawn to the relationship of polyploidy and aneuploidy, the latter is considered as a factor of genetic variability that may be an adaptive under the stress conditions[17-21]. Therefore it seems to be interesting to compare regularities of genome multiplication in different mammalian species in accordance with possible role of trophoblast cells in placenta formation.

THE WAYS OF SOMATIC CELL POLYPLOIDIZATION

At present a great number of data has been obtained that confirm the concept that modification (mostly, shortening) of the "archetypal" mitotic cell cycle results in genome multiplication[3,7,14,19,22-24]. Recently, there dominates a notion that such a modified cell cycle is characterized by alternating DNA synthesis (S) and Gap (G) phases in the absence of intervening mitoses, karyokinesis, and cytokinesis; a series of these shortened cycles allows cells to achieve high level of ploidy that may exceed 1000c[19,23,24]. Nevertheless, the extent of cell cycle shortening that results in polyploidization appears to differ significantly in different cell types and taxa. Therefore, it seems to be important to present short characteristics of different ways of polyploidization of somatic cells of different animals, plants, and human.

Switching off the last step of mitosis-cytokinesis-may be the first step to polyploidy. In this case, binucleate cell is formed (2c × 2). In the next cell cycle, both nuclei, as a rule, enter mitosis synchronously due to what the uniform metaphase plate is formed. If the mitosis comes to the end, it results in two mononucleated cells with tetraploid nuclei. Alternation of acytokinetic and following complete mitoses may result in formation of mono- and binucleate cells of the higher ploidy:

2c-(2c × 2)-4c-(4c × 2)-8c-(8c × 2)

Noteworthy, in this case, mitosis is the key event that allows formation of polyploid nucleus. Such a way of polyploidization was demonstrated basing on the dynamics of transition of mono- and binucleate cells with nuclei of different ploidy by using combination of cytophotometry and ^{3}H-thymidine DNA replication labeling[22]; this way was currently confirmed by using time-lapse video images[18]. Similar way of polyploidization was also demonstrated in some other mammalian cell types-cardiomyocytes[25,26] and aortic vascular smooth muscle cells[27].

Block of mitoses at the meta- and anaphase also may result in polyploidization: the cell do not complete the mitotic division owing to what the nucleus with the doubled number of chromosomes is formed[3,7]. Such a way of polyploidization recently is often described under the name of endomitosis[14,19,23]. However, taking into account participation of mitosis (although uncompleted) in this cell cycle, we adhere to the used term "restitutional" or "uncompleted mitosis"[3,28]. And now, in our opinion, it makes sence to unify the cases of uncompleted and acytokinetic mitoses under the term "reduced mitoses". Meantime, the term "endomitosis" includes prefix "endo" accounted for by the initial sence that implied chromosome/chromatid segregation inside the nucleus. Different forms of reduced mitoses may be observed simultaneously in the same cell type; in some cases they are accompanied by endocycles (see below); all these phenomena may result from disorder of many mitotic events. A good case in point is aortic vascular smooth cells studied by the time-lapse video[27]. In most cases there occurred normal anaphase chromosome segregation, but progress of cytokinesis was arrested; as a result, binucleate cells were formed. Sometimes such a binucleate cell renewed cytokinesis that came to the end with two daughter (probably diploid) cells formation. In other, rare, cases, mitoses resulted in mononuclear polyploid cells due to chromosome bridge(s) that did not allow forming two daughter nuclei. At last, there occurred some mitoses with shallow cleavage furrow and missegregation of sister chromatids; thereafter cleavage furrow disappeared and mononuclear polyploid cell was formed. All the phenomena were accompanied by downregulation of Survivin[27]; the disturbance of AuroraB/Survivin complex (a regulator of mitotic machinery) exerted such a pleiotropic effect on the progression of mitosis. The pleiotropic modifications of mitosis including endocycles (see below) were also observed in many cases of spontaneous and induced polyploidization, in particular, in endosperm and suspensor of higher plants[3,28], in cancer cells[29], in bovine trophoblast cells[11,30] and in some other cases.

The reduced mitoses in most cases can result in polyploidy of moderate level such as 4c, 8c, and some higher. As a rule, these modes of polyploidization allow cell to retain its mitotic potential[17,18]. In some cases they may result in "ploidy reversal" thereby generating diploid cells from the tetra- and octaploid ones as demonstrated by the time-lapse video[17,18,20].

More profound reduction of mitotic cycle results in endocycles. Originally this term covered all cases of genome multiplication accomplished without the nuclear envelope disappearance[3,5-7,28,31-33]. Therefore, it may be

suggested that in these cases, everything or nearly all stages of mitosis are reduced: the chromosomes do not form the metaphase plate, and nucleus retains the traits of interphase or prophase. The giant cells with polytene chromosomes of salivary glands of Diptera that probably represent numerous copies of the tightly attached elongated sister chromatids with clear-cut chromomere structure most probably are formed by means of block of mitosis in prophase.

The cycle of polytene nucleus devoid of mitosis was described in the giant trophoblast cells of mouse and rat. Using ^{3}H-thymidine labeling, two phases-S (endointerphase) and G (endoprophase) were identified in rat trophoblast cells. At the endointerphase (S-phase) the nucleus was filled with thin long paired Feulgen-positive threads, whereas at the endoprophase the bundles of non-classic polytene chromosomes were observed[5-7]. In mice, three phases of endoreduplication-G1, S, and G2 were discerned basing on the oscillative expression of the S-phase inhibitor p57^{kip2}[34].

The so-called classic endomitosis costs independently from the point of view of classifications used here. It is also a cell cycle devoid of phase of mitosis, although it includes phases of endoprophase, endometaphase, and endoanaphase, when the chromosomes attached to the nuclear envelope undergo condensation and splitting into sister chromatids followed by decondensation[3,7,31,35]. Interestingly, the nuclear envelope in most cases is retained throughout the whole cycle of endomitosis. Using the ^{3}H-thymidine delayed labeling, three phases of endointerphase-G1, S, and G2 were determined in the endomitotic cells of albumen gland of the snail Succinea lauta[36]. All the data, in our opinion, suggest that classic endomitosis, most likely, belongs to endocycles, *i.e.*, modifications of cell cycle, in which the genome multiplication is accomplished due to reduction of most of mitotic stages.

The classic and some other types of endomitosis are widespread mostly in invertebrates and plant tissues[3,28]. Meanwhile, such a mode of genome multiplication is observed in the human placental trophoblast[7,32,37,38], as well as in cancer cells[29,33,37]. The future investigation should elucidate the preferable meaning of the term "endomitosis".

Numerous data suggest that endocycles allow cells to gain very high ploidy levels. Thus, a series of endoreduplication cycle polytene nuclei of Drosophila salivary glands may result in 1024c[3,39-41] that most frequently provides irreversibility of switch from mitotic cycle to endoreduplication[22,23]. That is why, probably, endocycles are characteristic of the majority of invertebrate and plant differentiated tissues[3,28,42]. By contrast, the uncompleted mitoses including the acytokinetic ones in most cases can result in polyploidy of moderate level such as 4c, 8c, and some higher. As a rule, these modes of polyploidization allow cells to retain their mitotic potential-for example, in hepatocytes[17,18] that enable tissue to undergo an effective regeneration and sometimes (mostly in cases of experiments), to reverse to the diploid state[17,18].

POLYPLOIDIZATION IN DIFFERENTIATION OF THE RAT AND MOUSE TROPHOBLAST CELLS

Beginning from the onset of differentiation, the primary and secondary trophoblast giant cells (TGC) in rat and mouse placenta undergo a series of endoreduplication cycles that result in a very high ploidy level[1,4,6,43] and polytenization of giant nuclei. In the non-classic polytene nuclei, chromatin underwent condensation in the G-phase showing bundles of thick and short chromonemes under the nuclear envelope or near the nucleolus and decondensation in the S-phase where nuclei were similar to the interphase ones[5,6]. Later on, *i.e.*, after the 12th gestational day in rat, the features of non-classic polytenic chromosomes become less expressed that the precede transformation of the giant nucleus into polygenomic and multinucleate[5]. Switch to endoreduplication in the murine TGC is connected to the switch of the cyclin D isoform expression from D3 to D1; the arrest of mitotic cycle and the onset of endoreduplication was most probably accounted for by the failure to assemble the cyclin B/p34^{cdk1} complex during the first endocycle; in the subsequent endocycle the mitotic cyclin B was suppressed[43,44].

As distinct from the primary and secondary TGC, the junctional zone (JZ) trophoblast cells in rat and mouse placenta represent a highly proliferative cell population; the mitotic activity of JZ trophoblast cells persists up to midgestation[6]. Simultaneously the JZ trophoblast cells undergo differentiation into a number of cell subtypes involved in glycogen store, hormone production, invasion, *etc.*[9,45-49]. Unlike the secondary TGC that form a barrier at the border between semiallogenic maternal and embryonic tissues, the JZ trophoblast cells undergo polyploidization *via* uncompleted polyploidizing mitoses up to 8c followed by some endoreduplication cycles allowing them to reach 16-32c[6]. The DNA content measurement in the mitotic figures showed 4c, 8c and 16c mitotic figures indicating the ability of 2c-8c polyploid cells to undergo the complete mitotic division[6]. However, the polyploid mitotic figures often were of abnormal shape; chromosome bridges were frequent in polyploid anaphases, the double and multiple bridges were commonly present (Figure 1). Therefore, in this case, polyploidization increases the possibility of mitotic arrest that may result in interphase renewal leading to further polyploidization.

Difference in endocycle and uncompleted mitosis is illustrated by the data on their regulation by the transcription factor family E2F[24,50]. The canonical and atypical transcriptional programs converge to control the endocycle through the regulation of cellular events important for mitosis, karyokinesis, and cytokinesis. Thus, in the murine trophoblast giant cells (TGC) the targeted gene inactivation of *E2f1, E2f2*, and *E2f3* transcription induced abnormally large nuclei, their ploidy exceeding their characteristic ploidy levels 8c-256c. In the E2F7$^{-/-}$

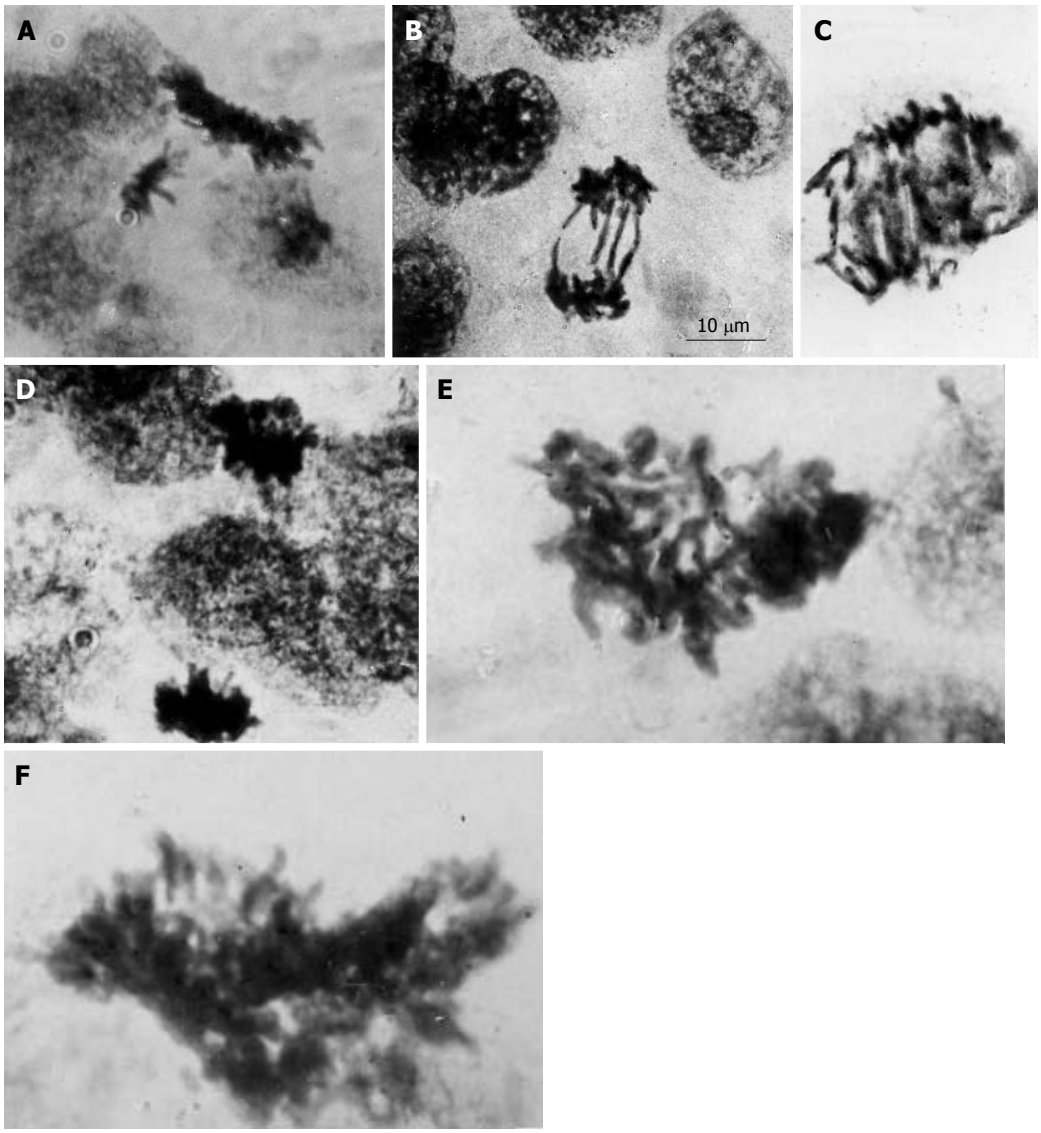

Figure 1 Diploid and polyploid mitoses in the junctional zone trophoblast cells in rat placenta at the 14th day of gestation. A: Tetraploid metaphase and diploid anaphase; B: Diploid restitutional anaphase with multiple chromosome bridges; C: Polyploid restitutional anaphase with multiple chromosome bridges; D: Tetraploid normal anaphase; E: Octaploid metaphase; F: Hexadecaploid metaphase.

and E2F8$^{-/-}$ an unusual number of mitosis at different phases were observed, which suggest interruption of endocycle[24]. The ploidy did not exceed 64c that, according to our data is normally characteristic of the trophoblast cells of rat placenta junctional zone undergone polyploidization *via* polyploidizing mitoses with subsequent endocycles[6]. This phenotype of E2F7$^{-/-}$ and E2F8$^{-/-}$ mice included upregulation of mitotic cyclins A2 and B1 in TGC as well as mitotic marker P-H3. In addition, up to 40% of nuclei underwent separation of two approximately equal nuclei out of the single giant nucleus[24]. The data suggest that the E2F7/E2F8 ablation promotes switch on mitotic cycle that restricts endocycling in TGC.

In liver, the combined ablation of *E2f1*, *E2f2,* and *E2f3* resulted in an increase of ploidy level[24] that suggests a possibility of hepatocyte ectopic endocycling. By contrast, ablation of E2F7/E2F8 leads to hepatocyte diploidization and to a decrease of the binuclear cell number. Thus, the canonical and atypical E2Fs exerts opposite effect: the canonical ones promote complete progression of mitosis, whereas the ancient, atypical one increases endocycling. Interestingly, study of the global gene expression in hepatocytes showed that majority of genes upregulated in E2F7/E2F8-deficient hepatocytes were downregulated in the E2F1/E2F2/E2F3-deficient ones, many of these genes had annotated to be the cell cycle function related, in particular, to bound to the G2/M transition or to mitosis[24].

Loss of E2F1 suppressed some, but not all mitotic defects in TGC and hepatocytes caused by E2F7/E2F8-deficiency. Ablation of mitotic cyclins in the E2F7/E2F8-deficient mice resulted in the greater ploidy in TGC, whereas hepatocytes in mice of similar phenotype showed similar ploidy as wild type mice. Thus, inhibiting the transcriptional network that signals G2/M progression or interfering with mitotic machinery (by cyclins A1/A2 ablation) reinstablished the mitotic block and reinitiated higher ploidy levels[24], probably, *via* endocycle

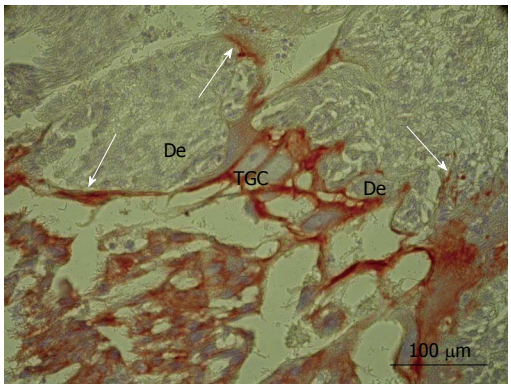

Figure 2 Secondary trophoblast giant cells of rat placenta at the 16th day of gestation. Note massive long cytokeratin-positive sprouts that embrace wide zones of decidual tissue (De). TGC: Trophoblast giant cells.

progression. The data demonstrate that an intricate E2F network involving balanced and antagonistic activities of canonical (E2F1-3) activators and atypical (E2F7/E2F8) repressors plays, most probably, one of the key roles in the mammalian endocycle control. Perhaps, most dramatic manifestation of altering the balance in the E2F network is ectopic mitoses in the *E2F7/E2F8* deficient TGC. Interestingly, such a phenotype was lethal, whereas inactivation of these genes did not produce any effect on the growth of liver. Thus, in the liver, mitosis is a prerequisite of polyploidization whereas in the highly endopolyploid murine TGC, in distinct from liver, such a key event is almost complete reduction of mitosis (*i.e.*, endoreduplication). It may be important for some functional peculiarities of these cell types: for example, retention of mitotic potential may be important for maintenance of regenerative ability of the liver.

Difference in the cell cycle machinery was also detected by using transcriptome analysis of the murine TGC and megakaryocytes[51]. The authors compared the expression level of orthologous genes in the DNA replication pathway and cell cycle gene controlling the G1-S transition and S-phase. The components of the DNA replication machinery including the origin recognition complex, minichromosome maintenance and proliferating cell nuclear antigen genes were strongly expressed in the TGC lineage. In contrast, polyploid megakaryocytes exhibited a reduction in the expression of these genes. The TGC similar to Drosophila polytene cells, on the contrary, showed a reduction in the expression of mitotic genes, as compared with diploid control embryonic cells[51], thereby confirming endocycle origin of their high level of ploidy[5,34,42]. Meantime, megakaryocytes showed increased expression of M-phase factors as compared to TGC or Drosophila[51]. Such a comparison suggests that genes controlling G1-S transition might play a key role in genome multiplication *via* endoreplication (in murine trophoblast and Drosophila salivary glands). By contrast, upregulation of mitotic events rather than S-phase factors play a key role in genome multiplication of megakaryocytes *via* endomitosis (reduced mitosis).

Significance of the two different ways of genome multiplication-endoreplication and reduced mitoses-can be shown on an example of functional organization of different trophoblast cell populations in the rodent placenta. According to our recent data, different trophoblast cell population show different patterns of intracellular cytokeratin localization. Long massive cytokeratin threads were found in the peripheral cytoplasm of the secondary TGC of rat and in their long sprouts by which they connected each other making continuous barrier at the border with decidua[7]. Similar cytokeratin immunostaining was observed in the trabecular spongiotrophoblast cells that line maternal blood sinuses. Clusters of low-ploidy proliferative and/or glycogen cells in the depth of JZ showed some weaker cytokeratin signal. Thus, the specific structure of giant trophoblast cells seems to provide a barrier between semiallogenic fetal and maternal tissues.

The east-european field vole Microtus rossiaemeridionalis provides another example of barrier function of TGC[52]. At the beginning of placentation TGC also form a continuous layer at the foeto-maternal interface. However, at midgestation, clusters of tightly attached low-ploid glycogen-rich junctional zone trophoblast cells progressively replace TGC thereby drawing them in the depth of the fetal part of placenta. Nevertheless, TGCs bound by their heavily cytokeratin-positive sprouts form a framework that holds other trophoblast cell populations and line lacunae with maternal blood.

Significance of specific TGC organization is confirmed by the data on the compound mutants on Cytokeratin 8 and 19[53]. In this case, there was an excessive number of TGC that however were not tightly attached to each other. Besides, $K8^{-/-}$, $K8^{-/-}/K19^{-/-}$ and $K18^{-/-}/K19^{-/-}$ knockout conceptuses died by the moment of placenta formation and showed placental hemorrhaging[54-56]. This apparently caused flooding directly to fetal tissue where these trophoblast cells normally separate embryonic blood from maternal circulation.

The enormous sprouts of TGC probably play another role in rat placenta. Some secondary TGC protrude the decidual tissue by means of large nippel-like highly cytokeratin-positive sprouts (Figure 2) that surround wide accumulations of decidual cells, the latter, probably, undergoing subsequent degradation[54]. This process was observed throughout the most of pregnancy and represents, most probably, a special kind of invasion that involves phagocytosis of decidual tissue by the trophoblast that may be called "group phagocytosis". The trophoblast phagocytosis that provides histotrophic nutrition of embryo was described in detail in the endothelio- and hemochorial placenta of a range of mammalian species[6,57-59]. Thus, in mice, processes of trophoblast giant cells penetrate a layer of uterine epithelial cells and internalize the cells[58]; phagosomes with fragments of decidual cells are observed in TGC. Recently, transcriptome analysis showed high expression of scavenger receptor class B, member 1 (scarb 1) required for phagocytic activity of TGC[51]. Therefore, gigantism of TGC

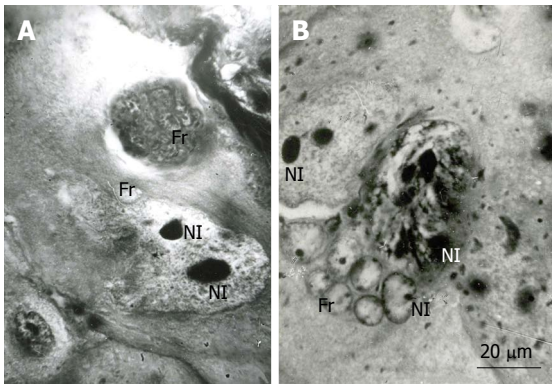

Figure 3 Fragmentation of the rat trophoblast giant cells. A and B: First the giant nuclei fall into two large nuclei, the latter then breaks down into numerous small fragments (Fr); B: The giant nucleolus falls down into several nucleoli (NI), then they form small nucleoli that seem to move into small nuclear fragments, some nucleoli in the nuclear fragments may be formed *de novo*. Heidenhain hematoxylin staining.

in this case probably allow them to perform invasion as well as partial degradation and phagocytosis of decidualized endometrium. It is interesting to note that one of the possible advantages of endoreduplication is that cell growth is accomplished without cell division that would imply a significant rearrangement of cytoskeleton[23]. Mitotic proliferation would prevent establishment of such a continuous system of the tightly attached phagocytosing TGC. Therefore, the endoreduplication allows TGC to combine growth at the restricted time period with formation of a barrier made of the tightly attached TGC that protect embryo from the immunological attack of the maternal organism and provide its histotrophic nutrition. It is also notable that the endoreduplication allows TGC growing without nuclear envelope disappearance. It also may be important, because isolation of genome inside the nuclear envelope may protect it from mutagenic effect of the degrading DNA of the phagocytosed maternal cells. In rare cases, in the field vole placenta we observed erythrocyte, *i.e.*, the anuclear cell, inside the phagocytic trophoblast cell undergone mitosis[6].

An interesting example of the necessity of endopolyploidy for the "barrier" function is the recently obtained data on subperineural glia (SPG) of Drosophila melanogaster by Italic. These glial cells were highly polyploid, and ploidy correlated with brain mass. Inhibition of the SPG polyploidy caused rupture of the septate junctions necessary for the blood-brain barrier. Thus, the increased SPG cell size resulting from polyploidization is required to maintain the SPG envelope surrounding the growing brain[60].

As to the trophoblast cell populations lying in the depth of the fetal part of placenta that first represent a proliferative pool of trophoblast, their primary steps of genome multiplication through reduced mitoses probably allow them to accumulate the great number of cells undergoing multidirectional differentiation into a number of subsets of cells within JZ and subsequently migrating into decidua (endovascularly and interstitially) as well as consisting trophoblast of labyrinth[6,7,61-63].

GENOME SEGREGATION: A TERMINAL STEP OF RODENT TROPHOBLAST GIANT CELL LIFESPAN

Beginning from the second half of pregnancy, the secondary giant trophoblast cells in rodent placenta undergo the so-called nuclear fragmentation when a part of giant nuclei break down into a number of the smaller nuclear fragments[5,7,64-66]. The fragmentation is preceded by depolytenization which is gained by disintegration of polytene chromosome bundles into the double chromonemes/endochromosomes; the process was described in detail earlier[5]. A similar process was recently investigated by using *in situ* hybridization the whole-chromosome labeling in the polytene chromosomes of Calliphora erythrocephala ovarian nurse cells[67]. In rat placenta, a portion of TGC undergoing nuclear fragmentation increases as the end of pregnancy approaches. Noteworthy, attenuation of DNA replication precedes nuclear fragmentation: this process begins in the nuclei that lost their capability for DNA synthesis[64]. Nevertheless, there were some reports about scarce murine ^{3}H-thymidine-labeled giant trophoblast cell nuclei undergoing fragmentation[66].

Interestingly, the process of nuclear fragmentation often begins with break down of the giant nuclei into two approximately equal parts (Figure 3). As a rule, one of these "parts" ("subnuclei") undergoes more complete breakdown into numerous fragments. It is notable that ablation of mitosis progression regulators E2F1, E2F2, and E2F3 often resulted in break-down of mouse trophoblast giant nuclei into two approximately equal parts[24]. This suggests that although the process of giant nuclear segregation is not similar to mitosis, most probably, some elements of the mitotic machinery are involved in this mechanism.

Cytophotometric and cytofluorometric measurement of DNA content measurement showed a tendency to the whole-genome distribution of DNA into the nuclear fragments (Figure 4). In the rat secondary TGC, the histograms show quite clearly distinguishable peaks corresponding to different ploidy classes, *i.e.*, 1c, 2c, 4c, 8c, 16c, and 32c. However, there occurred incidence of some intermediate values of DNA content that could not be explained by the DNA synthesis, because in the nuclear fragments the S-phase is absent. In the mouse secondary TGC the tendency for the whole-genome distribution was also observed, but some peaks tend to be 3c and 6c values[5,64]. Similar data were obtained on the giant cells of the rabbit trophoblast[5].

The DNA content measurement of nuclear fragments of the field vole secondary giant trophoblast cells showed more clear-cut correspondence to the distinct ploidy classes multiple to 2c[5,68].

Behavior of natural chromosome markers, *i.e.*, sex chromatin body and nucleolus, were observed in the giant nuclei undergoing fragmentation. The inactivated X-chromosome that forms a condensed chromatin body in the interphase nucleus was undergone to successive

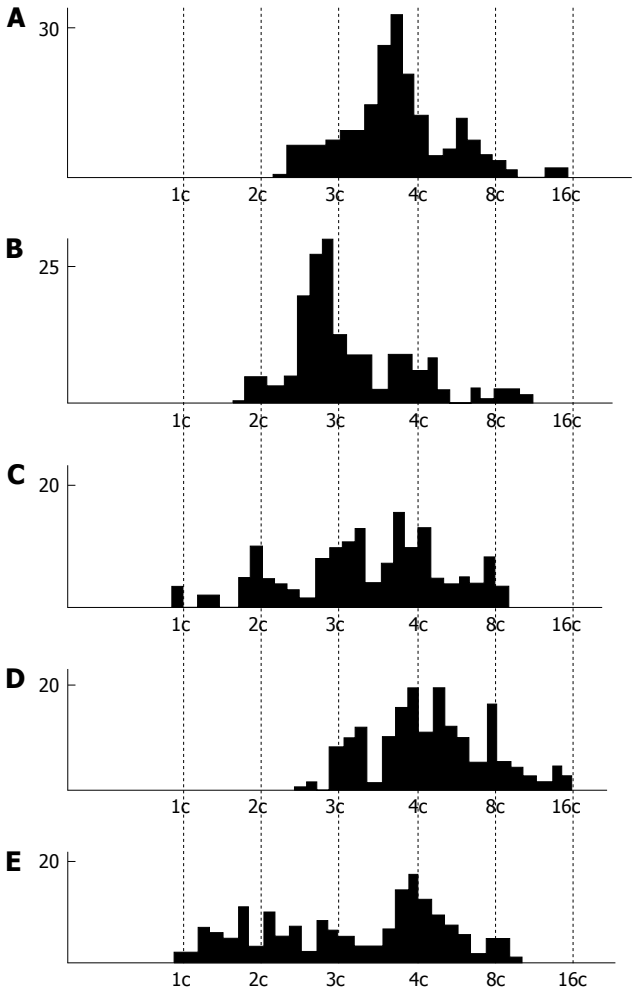

Figure 4 DNA content in the nuclear fragments of secondary trophoblast giant cells of mouse (A and B) and rat (C-E) at the 17th (A), 19th (B), 14th (C), 16th (D), and 18th (E) day of gestation. Abscissa: The DNA content (arbitrary units, logarithmic scale), and ploidy, c; Ordinate: the number of nuclear fragments.

doubling in each cycle on endoreduplication of the rat secondary TGC of female embryos[1]. In the course of fragmentation, each nuclear fragment obtained a small condensed sex chromatin body attached to the nuclear envelope[5,65]. Similarly, each fragment contains a small nucleolus. At the onset of fragmentation, when the nucleus breaks down into several rather large fragments, the large nucleoli also break down into several ones that seem to be converted into separate fragments (Figure 3). Nevertheless, as to the final steps of fragmentation, it is not easy to decide whether the small nucleoli came from the initial giant nucleus or were synthesized *de novo*.

In the mouse secondary TGC, polytene chromosomes were characterized by the presence of large clear-cut heterochromatin blocks attached to the nucleolus (Figure 5). In the course of nuclear fragmentation, the heterochromatin blocks were separated into small blocks that were observed in the nuclear fragments near the nucleolus (Figure 5).

To determine whether the separate chromosomes are distributed into the nuclear fragments in correspondence with ploidy level, we used the so-called gonosomal chromatin bodies (GCB) in the secondary TGC of the field vole Microtus rossiaemeridionalis by Italic that represent large heterochromatin blocks of X and Y chromosomes that form prominent condensed chromatin bodies in the interphase nucleus. According to the DNA content measurement in the nuclear fragments of the secondary TGC and their GCBs, each fragment contains GCB(s) in correspondence with its ploidy level[8].

The above-mentioned data confirm that isolation of small "nuclei" inside the TGC is a process in which single chromosomes are regularly dispersed into nuclear fragments. Nevertheless, the data of cytophotometry leave open the possibility of some aneuploidy in the nuclear fragments. Thus, we can conclude that polytene nuclei of TGC are transformed into the multinucleate giant cells, each of nuclei contains the euploid (1c, 2c, 3c, 4c, *etc.*) or the near-euploid set of endochromosomes.

The nuclear fragmentation seems to be a rare common process, and its mechanism is not elucidated in detail. Active participation of membranes of the nuclear envelope and its derivatives was observed in the rat secondary TGC[64]; bundles of intermediate filaments were also observed in invagination of nuclear envelope of nuclei into the process of fragmentation. However, such a process, most probably, makes possible separation of one or more genomes into separate fragments, and their number is not always divisible (2^n)c.

In the literature, very little data are known to draw analogies that could make an explanation of the above-mentioned chromosome distribution in nuclear fragments. Thus, in endomitotic nuclei of the seminal follicle wall of locust separation of some endochromosome groups corresponding to haploid, triploid, diploid, and hexadecaploid chromosome sets[69], *i.e.*, segregation of a number of genomes not multiple to (2^n)c. The data also confirm the possibility of the spatial separation of genomes without their entering mitosis.

Another example of genome segregation is observed in polyploid nuclei of protists. In some Radiolarians, nuclei are polyploid, and nuclear division is achieved *via* genome segregation[70]. For example, in Aulacantha by Italic, there is a single polyploid, the so-called primary nucleus. In the course of nuclear division, a great number of large chromosomes are presumably gathered into chains. During endomitosis that precedes division, reproduction of the "gathered" chromosomes takes place. In the course of sporogenesis, fragmentation of the primary nucleus results in a number of secondary nuclei; the latter initially lie in the same cytoplasm, but later on the cytoplasm also undergoes subdivision into a number of secondary bolls. Then the secondary nuclei undergo a series of division and, as a result, the cytoplasm breaks down into mononuclear prespores that divide once again to give rise to zoospores. All this complicated process is considered as a breakdown of the primary nucleus into separate genomes (*via* their segregation) and depolyploidization is carried out in several steps.

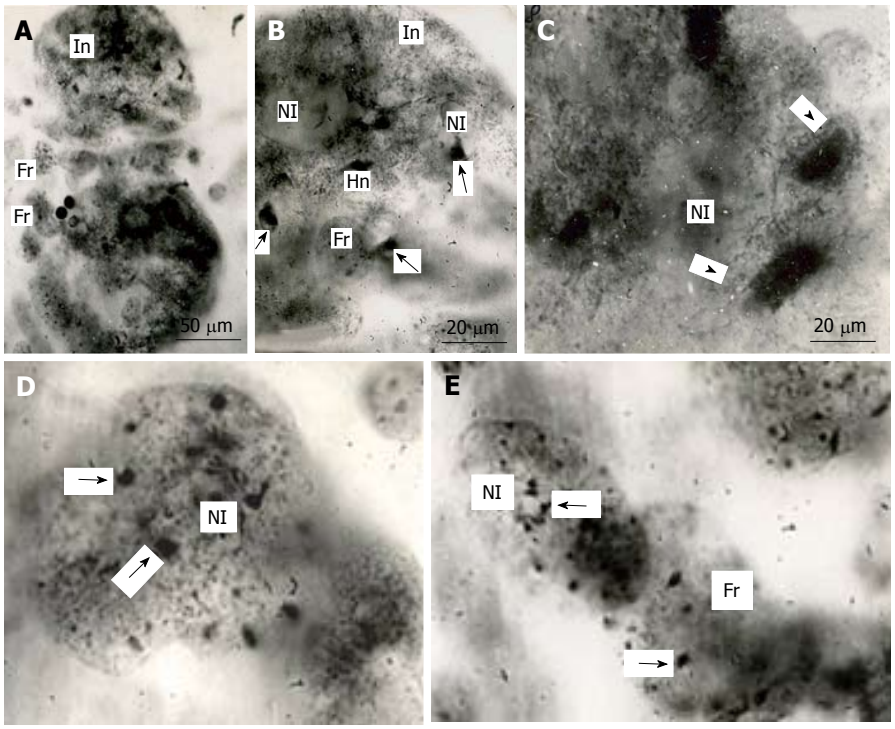

Figure 5 Distribution of heterochromatin blocks into nuclear fragments of mouse trophoblast giant cells. A: The initial nucleus (In) of trophoblast giant cells (TGC) in the process of fragmentation; B: The initial nucleus contains large clear-cut heterochromatin blocks (Hn, arrows) near nucleoli (NI); C: The nucleus contains non-classic polytene chromosomes with numerous distal loops (arrowheads); D and E: Nuclear fragments with small heterochromatin blocks (arrows) near nucleoli. Squash preparations, aceto-orcein staining. Fr: Nuclear fragments.

Since placenta is a provisory organ, it is felt that there should exist a mechanism of death of its cells, especially the peripheral ones) as the term approaches; it would enable placenta to separate at birth. In fact, the lifespan of the secondary rat TGC is strictly 22 d, which coincides with the length of pregnancy; it is not changed in culture and under conditions of transplantation under the kidney capsule[4].

The multinucleate secondary TGC in rat placenta show some signs of apoptosis, attenuation of cell functions and degradation: condensation of chromatin located under the nuclear envelope of the nuclear fragments, inactivation of nucleolus, destruction of cytoplasmic organelles, *etc.*[5,7]. Nevertheless, the outer nuclear membrane of some nuclear fragments generates outgrowths continuous with the agranular endoplasmic reticulum, which, in turn, produces the double-membrane channels that delimit cytoplasmic mic territories around nuclear fragments. It is notable that the territories contain the whole set of cell organelles-mitochondria, Golgi complex, granular and agranular endoplasmic reticulum, numerous polysomes. In many cases, nuclear fragments with their cytoplasmic territories that can be called "subcellular compartments", look quite viable; they are often isolated from the rest of cytoplasm of TGC that show signs of degradation. Moreover, sometimes this process results in isolation of subcellular compartments from other ones; however, in some cases the compartments connect each other by means of typical intercellular junctions (Figure 6). Occasionally it is possible to see as the pseudopodia formed by one subcellular compartment as though try to surround another one (Figure 6). Therefore, the subcellular compartments of TGC may behave like full value cells[7]. Meantime, in other cases, these compartments look apoptotic.

Thus, as the term approaches, TGC undergoing attenuation of reproductive, transcriptional, and other function simultaneously separates numerous subcellular compartments with near-euploid nuclei; the compartments may be both viable and apoptotic. It seems to be important that these fragments lose completely their capability for genome reproduction that would prevent renewal of proliferation of trophoblast cells. Nevertheless, the subcellular compartment formation may represent a reserve mechanism that preserve trophoblast genome for unknown functions. Further investigation may probably shed light on the significance of this phenomenon.

POLYPLOIDIZATION OF SILVER FOX

In the silver fox the trophoblast invasiveness is manifested in other form as compared to rodents. In Carnivores, syncytiotrophoblast only partially destroys uterine epithelium and comes into contact with blood vessels, without destroying endothelium[71,72]. The main part of fox placental trophoblast in which active proliferation and polyploidization takes place is out of contact with the glandular zone of endometrium and forms the fetal part of placenta[73]. The giant trophoblast cells are scattered throughout the fetal part of placenta (Figure 7), forming

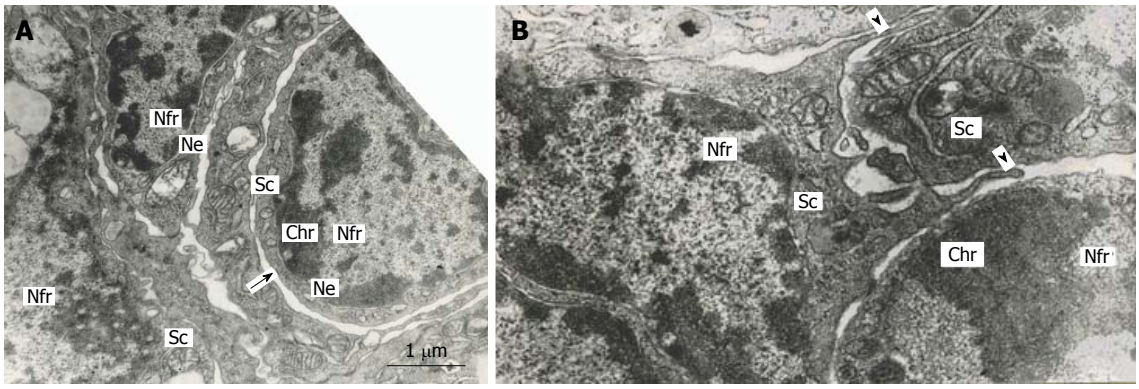

Figure 6 Subnuclear compartments in the rat trophoblast giant cells are separated from each other by forming rather wide channels of endoplasmic reticulum (A, arrow); some of them show intercellular junctions (A and B), some compartments (B) produce pseudopodia-like outgrowths (arrowheads) moving to other compartments. Nfr: Nuclear fragments; Chr: Condensed chromatin; Sc: Subnuclear compartments; Ne: Nuclear envelope.

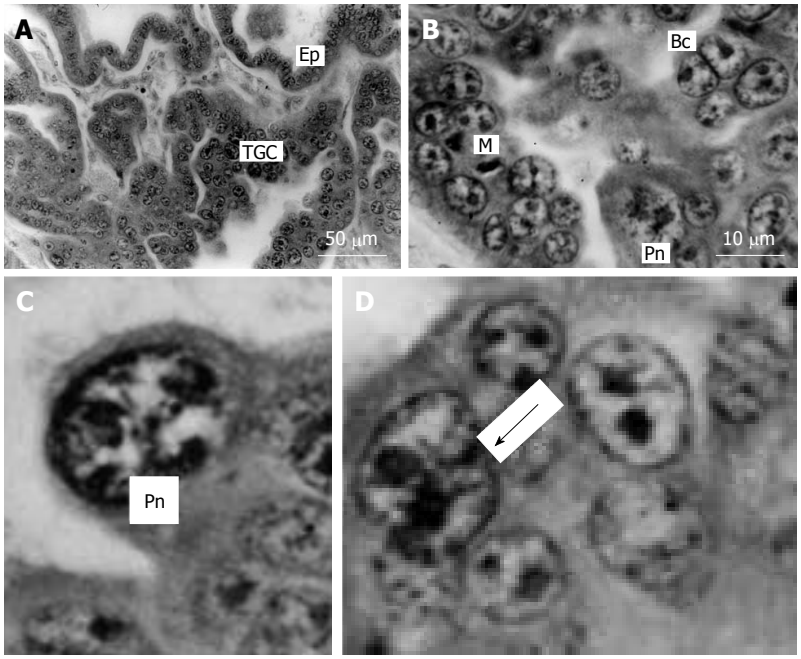

Figure 7 Silver fox placenta. A and B: Trabeculae of trophoblast and folds of uterine glandular epithelium (Ep) mutually contact each other, trophoblast giant cells (TGC) are scattered in the fetal part of placenta between accumulations of proliferative cells; B: Mitotic (M), binucleate (Bc) and cells with polytene nuclei (Pn); C: Nucleus with non-classic polytene chromosomes; D: A polyploid nucleus in the beginning of fragmentation (arrow). Meyer hematoxylin staining.

the largest accumulation near absorptional zones.

DNA content in the trophoblast cells corresponds predominantly to 4c-64c, the main peaks lying at 16 c and 32c; meantime, the highest ploidy corresponded to 64c[73]. Therefore, the ploidy level of the silver fox is lower as compared to the giant trophoblast cells in rodents. Another peculiarity is that there was a considerable deviation from (2^n)c, with a tendency to $2n \times 3$c values and a great variety of the intermediate values suggesting a significant incidence of aneuploidy[73].

Dynamics of polyploidization during placentation showed several steps of increase and decrease of ploidy levels. At the 20th day of pregnancy the trophoblast nuclei reach the highest ploidy levels (Figure 8). At the next, 21st day, percentage of nuclei of higher ploidy levels decreases. This trend is also seen at the 22nd day: the "divergence" of ploidy is observed, the percentage of diploid and tetraploid nuclei rises to 10% and 20%, respectively; simultaneously the percentage of 32c and 64c nuclei also increases as compared to that at the 21st day, and trophoblast cell population reaches the highest ploidy levels-128c and 256c. At the 23rd day, a new "wave" of polyploidization, similar to 20 d, takes place. The reason of such a fluctuation may be accounted for appearance of new zones of trophoblast cell proliferation. Therefore a part of trophoblast cells divide mitotically providing diploid and low-polyploid cells whereas other cell undergo endocycles to reach high ploidy levels. Simultaneously, pictures similar to nuclear fragmentation described in the rodent giant trophoblast cells (see above) were observed

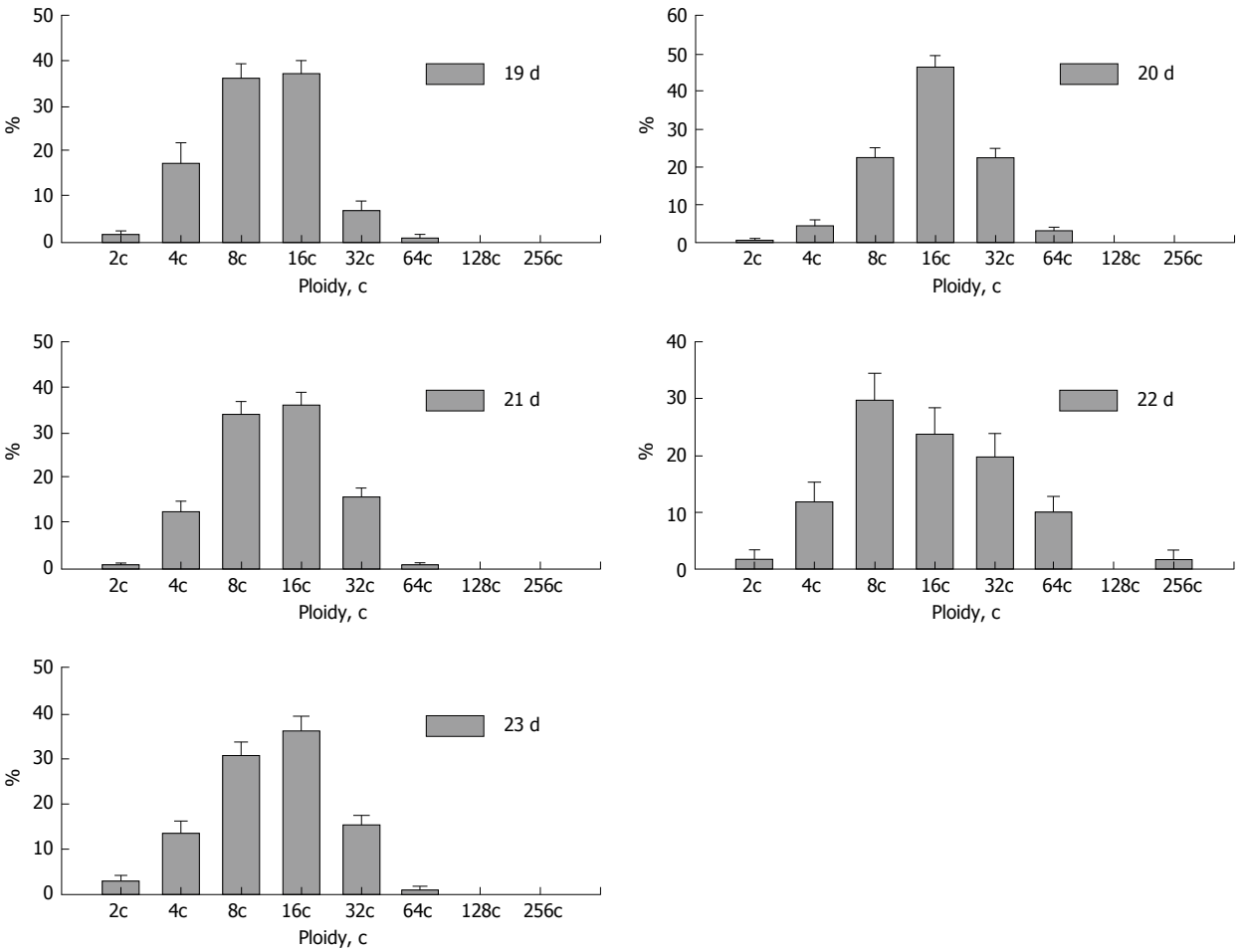

Figure 8 Dynamics of polyploidization of the trophoblast cells in silver fox placenta.

in the fox trophoblast[73]; such a genome segregation also may be a reason for a decrease of the ploidy level.

The DNA content measurement in mitotic figures showed 4c, 8c, and 16c mitotic figures that confirm the ability of 2c-8c to divide mitotically. The presence of binuclear cells, polyploid mitoses, and atypical metaphases and anaphases including multipolar mitoses indicates acytokinetic and uncompleted mitoses as a way by which the cells reach low (4c-8c) ploidy levels[6]. The higher ploidy levels may be attained by switch to endoreduplication cycle; it is confirmed by the presence of nuclei with characteristic non-classic polyteny (Figure 7). Interestingly, histograms of DNA content in the mitotic figures also show a tendency for "triploidy". It should be noted that signs of aneu- or/and triploidy are intrinsic of fox trophoblast: the uterine epithelial cells that also polyploidize reaching 4c-8c show quite euploid DNA content histograms[73].

The reason for deviation from euploidy in the fox trophoblast cells was not clarified completely. However, it cannot be ruled out that it may result from depolyploidization as a result of the multipolar mitoses and the process similar to the nuclear fragmentation described in the previous chapter.

PECULIARITIES OF HUMAN TROPHOBLAST GENOME MULTIPLICATION

Human trophoblast shows, at the first glance, a great difference in the trophoblast polyploidization as compared to rodents, which probably is accounted for by human placenta formation and growth characteristics. During the most of pregnancy, the trophoblast continuous layer at the border of decidua consists of a layer of syncytiotrophoblast, whereas several local zones of the intrauterine invasion of extravillous trophoblast (EVT) are concentrated at tips of the anchoring villi[74]. Numerous data of caryologic analysis as well as DNA flow cytometry showed the prevalence of the diploid or, sometimes, near-diploid chromosome set in the human trophoblast cells[75-77]. Meantime, the human trophoblast invasion show many analogies in regularities of the genome reproduction to trophoblast of rodent placenta.

Thus, in human, like in rat placenta, the deep intrauterine interstitial and endovascular invasion is accomplished at the complete cessation of DNA replication[5,44,74,78]. Lack of genome replication of the invading

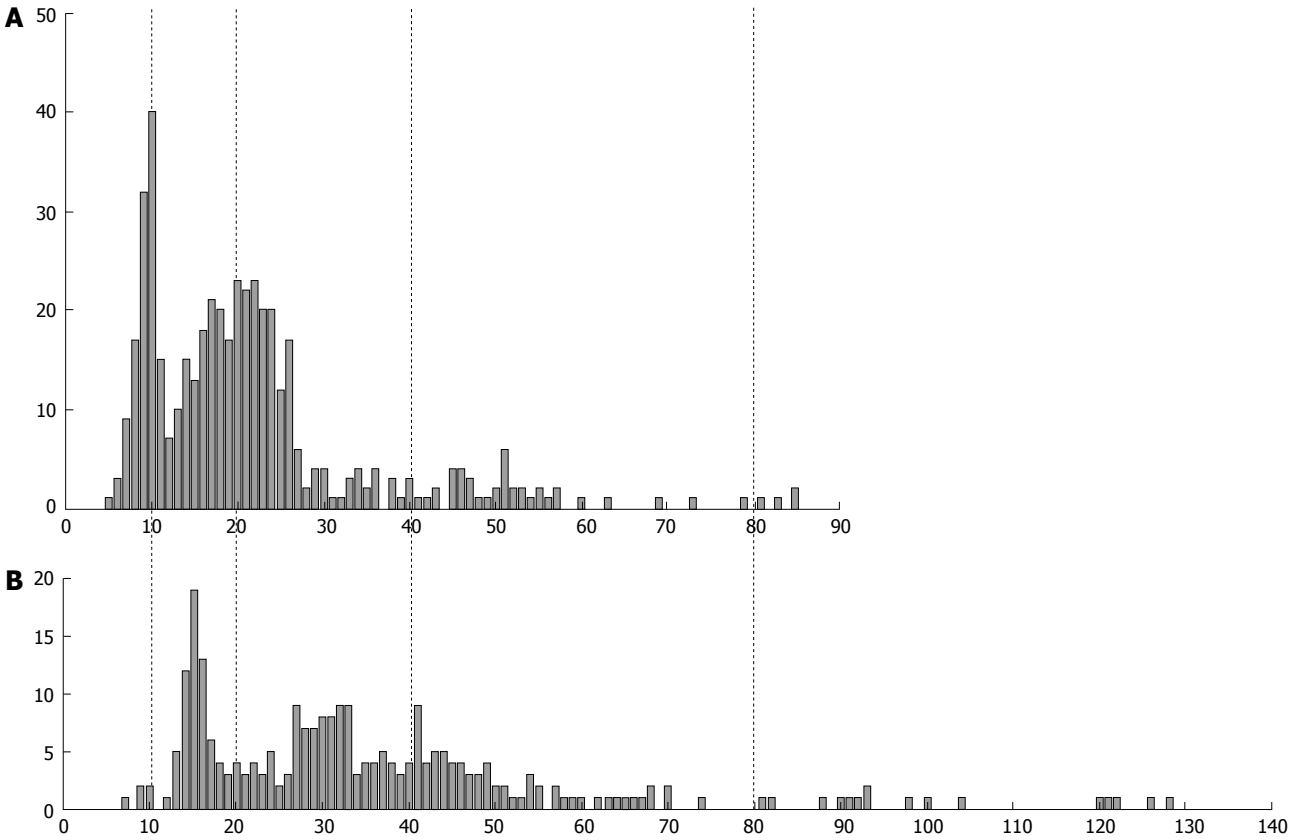

Figure 9 The difference in DNA content in human extravillous trophoblast cells invaded endometrium in placentae of the first trimester between two individuals (A and B). Abscissa: The DNA content (arbitrary units); Ordinate: The number of cells.

trophoblast may prevent the ectopic proliferation of the trophoblast cells both within the uterus and in other parts of the maternal organism.

Polyploidization of the extravillous trophoblast cells also show similarities with rodent placenta. Thus, the proliferative EVT attached to the basal membrane of tip of villi are mostly diploid, but the ploidy increases progressively to 4-8c in process of approach to the border of decidua[7,79]. Thus, like in rat, mouse, and field vole, the highest ploidy is characteristic of the human trophoblast cell layer that borders the semiallogenic maternal tissues.

An interesting peculiarity of the human EVT genome reproduction is a ploidy divergence that takes place at the moment of intrauterine invasion (Figure 9)[7,79]. The fraction of 8c cells increased by up to 9.7%; besides, a few 16c nuclei appeared, whereas, on the other hand, the percentage of diploid cells also rise noticeably-on average, from 20% to 30%. Such a tendency was also found in the EVT invaded up to myometrium: the percentage of diploid nuclei exceeded 40%, whereas a number of highly polyploid 8c and 16c nuclei persisted in this zone.

One of peculiarities of the trophoblast cell polyploidization is a great variety of the genome multiplication between individual placentae[79]. Thus, Figure 9 presents histogram of the DNA content distribution in the extravillous trophoblast cells invading decidualized endometrium in placentae of two pregnant women. We can see that one of them shows more or less clear-cut peaks corresponding to 2c and 4c; the latter ploidy class prevails, some cells being able to reach as many as 16c. Another sample shows more extended histogram, in which the main peaks tend to 3c and 6c, the greatest number of cells being located between 4c and 8c. Besides, in the second sample, the noticeable number of cells exceeded 16c. A correlation between DNA content values not multiple to (2^n)c and the highest ploidy level is seen here; besides, a tendency for 3c × $2n$ values is characteristic of the samples of the higher ploidy level.

Endoreduplication of human cervical trophoblast with prevalence of tetraploid cells was also found by means of caryological analysis with fluorescent *in situ* hybridization (FISH) staining of the chromosomes X, Y, and 21[80].

Endocycle progression in the human EVT is confirmed by downregulation of mitotic cyclins A and B1 alongside the invasion pathway and peculiar expression of Cyclins D and E as well as $p57^{kip2}$[7].

According to our data, a great number of DNA content values not multiple to (2^n)c is observed in the beginning of the EVT invasion. When the cells penetrate endometrium and myometrium, more clear-cut peaks tending to (2^n)c are observed[7,79].

Interestingly, some data suggest the necessity of aneuploidy for normal EVT trophoblast invasion. Thus, molecular cytogenetic data showing that approximately 20% to 60% of interphase EVT invasive cells in the normal

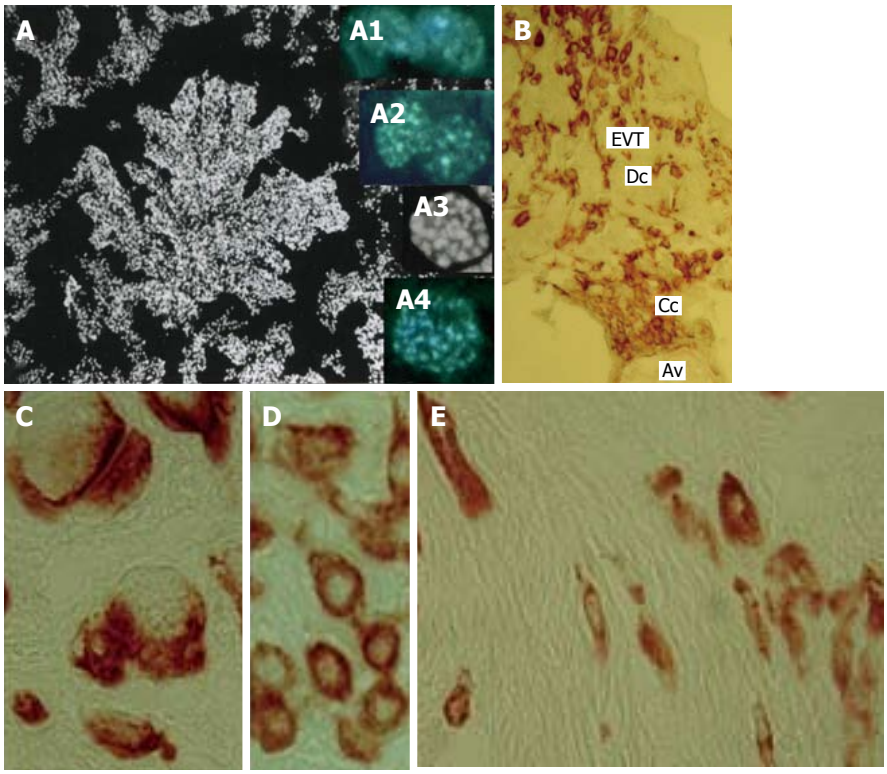

Figure 10 Human trophoblast cells undergoing polyploidization. A: A squash spread of chorionic villus stained with 4',6-diamidino-2-phenylindole; A1: Binucleate cell in interphase; A2: Binucleate cell in prophase; A3: Endometaphase; A4: Endoanaphase; B: A cell column (Cc) at the tip of anchoring villus (Av) generates a pool of extravillous trophoblast cells (EVT) capable for invasion of decidualized endometrium (Dc); C: Multinucleate EVT invaded decidua; D: Interstitial EVT of moderate ploidy; E: Small elongated low-ploidy EVT that reach myometrium.

pregnancies acquired aneusomies involving chromosomes X, Y, or 16[81]. The incidence of aneuploidy positively correlated with gestational age and differentiation to the invasive phenotype. Scoring 12 chromosomes in flow-sorted cytotrophoblasts showed that more than 95% of the cells were hyperdiploid. Thus, aneuploidy appears to be an important component of normal placentation, perhaps limiting the proliferative and invasive potential of cytotrophoblasts within the uterus[81].

A series of recent investigations allow some authors to put forward a concept that transition from endoreduplication to polyploidy and then to aneuploidy represents a genetically diverse pool of cells[18-20]. The authors suggest that a set of these genetically changed cells may be useful and amplified under conditions of stress[18]. In the case of invading trophoblast we can assume that aneuploidy that accompanies the complicated processes of the trophoblast genome reproduction may rise several genotypes that may promote cells to survive under the stress conditions inside semiallogenic maternal tissues. It may be suggested that the most stressful condition for EVT cells is a moment of overriding the border with decidualized endometrium; at this moment the optimal genotypes are selected; later on, deviation of the euploidy decreases. It cannot also be ruled out that simultaneously the cells of higher ploidy are selected to invade the proximal part of endometrium and the small low-ploidy elongated cell to invade deep up to myometrium (Figure 9).

It is not easy to explain the possible significance of tendency to triploidy in the invading human EVT (that may be analogous to the same tendency observed in silver fox). Triploidy of human trophoblast was reported in several papers[76,82]. Very often, it is observed under pathological conditions connected with ectopic or disturbed trophoblast invasion, for example, in the hydatidiform mole[76] or severe preeclampsia[82]. Meantime, basing on the above-mentioned data on the possibility of non-mitotic genome segregation (nuclear fragmentation) as a regular step of trophoblast cell lifespan, we can suggest that such a process may result in separation of the chromosome set non-multiple to (2^n)c. This process, most probably, does not include the way of exact distribution of all chromosomes into the daughter cells characteristic of mitosis, that is why it may be suggested that such a way of depolyploidization of trophoblast cells may result in aneuploid genotypes.

The reason for such a variability may be accounted for by the ways of human EVT polyploidization. Endomitosis and non-classic polyteny were observed in the trophoblast cells in normal human pregnancies[32,37,83] as well as in hydatidiform moles[32].

According to our data, the processes like endoreduplication or/and endomitosis, most probably, prevail in the EVT invasive pathway. The Figure 10 shows a chorionic villus tip stained with DAPI. A great number of cells with numerous chromocenters as well as nuclei at different stage of endomitosis are present there (Figure 10)[38]. Meantime, a number of nuclei with enlarged chromo-

centers, whose number was not increased with increasing ploidy, suggest a possibility of passages of several rounds of polytenization[38]. Thus, it cannot be ruled out that a relatively low ploidy level in the human placenta prone to aneuploidization may be linked with endoreduplication/endomitosis that, theoretically, may be involved in single/double/triple genome segregation processes.

GENOME MULTIPLICATION IN THE TROPHOBLAST CELLS OF RUMINANTS

Ruminants represent a mammalian group with the so-called epitheliochorial placenta, in which trophoblast invasiveness is minimal: the trophoblast cells attach to the uterine epithelium mostly without its degradation[84]; meantime, the trophoblast cells express the same integrins as the highly invasive human trophoblast cells[85]. The bovine trophoblast cells that come into close contact to the uterine epithelium retain their mitotic activity and reach the ploidy level 4c-8c *via* reduced (restitutional) mitoses[11,30]. Tripolar mitoses suggesting a possibility to reverse to the lower ploidy level also are present there. It is quite probably that lack of deep invasion that does not imply phagocytosis of epithelium, blood or decidual cells by the trophoblast does not require switch to the endoreduplication cycle, although a low level of ploidy is still necessary for such a mode of feto-maternal interaction.

The most striking example of polyploid mitoses was described in alpaca trophoblast. Elevated nuclear DNA contents in the giant trophoblast cells of alpaca could be achieved by modified cell cycles with a complete lack of mitosis (endoreduplication) or with incomplete mitoses[12]. Electron microscope observation made on serial sections revealed that TGCs are truly multinucleate with several highly lobulated nuclei. Feulgen staining showed that TGC nuclei have the higher DNA content than nuclei of other trophoblast cells. The number of argyrophilic nucleolar organizer regions in nuclear profiles of TGC was between 15 and 100; numerous nucleoli suggest polyploidization, in which mitoses take part, as this was observed in the rat decidual cells[86]. In the latter case, numerous decidual cells are formed by active mitotic divisions; numerous binucleate cells were observed, there were many enlarged mitoses of irregular form, a part of mitoses were tri- and tetrapolar. Very often, bi-tri- and tetranuclear cells were observed that could result from multipolar mitoses, the nuclear ploidy reached 8c. Numerous nucleoli (up to 20) were also observed in the lobulated nuclei. As to alpaca trophoblast, even larger multipolar mitotic figures with maximal diameters of 80 μm were observed in placentas on gestation days 264 and 347. No cytokinesis was seen in TGC[12]. The authors note that subsequent acytokinetic mitoses may lead to accumulation of chromosomes and centrioles in TGC. With increasing ploidy levels, the shape of these polyploidizing mitoses becomes more irregular. The restitution of nuclei after these complex multipolar mitoses is likely to result in the irregular nuclear shape in TGC. Therefore, it is an exceptional example of polyploidization *via* restitutional (reduced) mitoses that probably may lead to the high ploidy level. It seems doubtful that in this case the multipolar mitoses may result in depolyploidization.

Thus, a conclusion can be made that shallow-invasive trophoblast cells of ruminants involve polyploidization *via* restitutional (reduced) mitoses.

CONCLUSION

The data considered here demonstrate that trophoblast cells of different mammalian species are characterized by different modes of multiplication of their genome that, probably, is linked with their ploidy level, capability for further proliferation, necessity of irreversible or, on the contrary, of reversible polyploidization that, in turn, most probably, is accounted for by the trophoblast cell specific function.

One of the most important advantages of polyploidy for the trophoblast cells contacting semiallogenic maternal tissues may be the delay of proliferation to avoid segregation of the damaged chromosomes[19]. Besides, multifold genome doubling makes the endocycling cells more resistant to mutagens. Thus, the highly endopolyploid murine TGC are much more resistant to irradiation than the low-ploid trophoblast cells[42]. Interestingly, endoreduplication as a response to mutagens can be induced experimentally, and some regulatory pathways were recently revealed. Thus, following double strain breaks induction in the root tips of Arabidopsis by Italic the cells switch to endoreduplication[87]. This cell alteration requires the plant-specific transcription factor Suppressor gamma response 1 which transmits signals from the conserved Ataxia Telangiectasia mutated and Ataxia Telangiectasia-mutated and RAD3-related DNA damage sensor kinase[86]. This DNA break response produces transcriptional changes that are consistent with downregulation of mitotic factors and upregulation of cell cycle genes that promote endoreduplication.

Recently there were obtained some other confirmations of significance of non-mitotic polyploidization under condition of DNA damage. In Drosophila by Italic, endoreduplication cells acquire resistance to DNA damage through a mechanism involving the silencing of cell death genes[19,88]. Similarly, endoreduplication mouse trophoblast cells that undergo endoreduplication also downregulate the DNA damage response. During differentiation of trophoblast stem cells into polyploid TGC, the protein level of damage-responsive Chk1 is decreased providing for endoreduplication. This decrease in Chk1 enables polyploid trophoblast cells to evade apoptosis through suppression of the DNA damage pathway[89,90].

It seems to be obvious that there are two main ways of genome multiplication: endoreduplication that involves downregulation of mitotic events and reduced mitosis ("endomitosis") in which entrance into mitosis is a prerequisite of genome multiplication. Endoreduplica-

tion allows to combine growth and specific functioning of cells that retain their peculiar organization. On the contrary, the cells that polyploidize *via* reduced mitoses retain their mitotic potential necessary, for example, for accumulation of a great number of cells, or for regeneration. In case of rodent trophoblast, the highly polyploid TGC undergoing endoreduplication serve a barrier between semiallogenic tissues whereas highly proliferative low-ploid trophoblast cell populations give rise to numerous JZ and labyrinth trophoblast cells.

Endoreduplication is a characteristic of highly invasive trophoblast cells. It is confirmed by comparison of placentation of mammalian species. Thus, highest invasiveness is characteristic of rodent and human placenta in which invasive trophoblast cells undergo endoreduplication whereas reduced mitoses are observed in the low invasive epitheliochorial placenta of ruminants.

Another advantage of polyploidization that is widely discussed now is a possibility to gain a variety of genome changes. Such a possibility was recently demonstrated in hepatocytes[17,18,21]. Apart from polyploidization by acytokinetic and subsequent normal (polyploidizing) mitoses, hepatocytes may undergo depolyploidization that result from multipolar mitoses[18]. Indeed, during multipolar mitosis, microtubules from different poles of spindle can be attached to a single kinetochore, and failure to repair such merotelic attachment can lead to incomplete chromosome segregation. In this case, chromosome bridges and laggings were observed. Karyotype analysis showed high frequency of aneuploidy in the normal murine liver: nearly 25% of hepatocytes from the 3-wk old mice were aneuploid, the aneuploidy increasing to 70% in the 4-15-mo old mice[18]. Interestingly, the entire chromosomes were gained and lost in this case, and structural rearrangement was rarely seen; besides, all chromosomes of genome were affected equally. One or more chromosomes were gained or lost by each aneuploid hepatocytes, and occasionally chromosome gains balanced losses mimicking the normal chromosome number. That is why, probably, aneuploidy did not lead to noticeable deviation from (2^n)c seen in histograms of the DNA content distribution in hepatocytes measured by DNA cytometry[91]. The karyotype and FISH analysis also revealed a significant level (30%-90%) of aneuploidy in the human liver[17]. Strikingly, gain and loss of chromosomes in hepatocytes under stress conditions may result in selection of the specific karyotype that can result in adaptation to injury[20].

The statement that polyploidy is often accompanied by aneuploidy may be accounted for by the fact that aneuploidy often results from depolyploidization that, in turn, requires polyploid cell formation. Meantime, it should be kept in mind that genome multiplication, in many cases, is irreversible. In this connection, a spectacular example is given by the ovarian follicle nurse cells[42]. When the cells reach 32c, centrioli leave their position near nucleus and move into oocyte through the cytoplasmic bridges. This is a way by which the cell "burns the bridges" to ploidy reversal and renewal of mitotic divisions. In the majority of cells normally undergoing endocycles, the genome multiplication is irreversible[22]. Therefore, aneuploidy may arise in the modified cell cycles prone to depolyploidization. Actually, at present little is known as to which cell types may be capable for the spontaneous non-pathologic aneuploidization.

Endopolyploidization as an escape route from cell death has been investigated on cancer cells. It has been demonstrated that after irradiation the great majority of cells die due to a mitotic catastrophe[92]. However, some cells escape the mitotic catastrophe and polyploidize. A few endopolyploid cells undergo depolyploidization and create a set of para-diploid viable cells capable for mitoses that may give rise to subclones[92,93]. Recent data of this research group indicate that tumor cells can induce opposing processes of senescence and stem cell generation in response to these treatments whose biological significance and molecular regulation currently are poorly understood[94]. Although cellular senescence is typically considered a terminal cell fate, it was recently shown to be reversible in a small population of polyploid cancer cells induced after DNA damage. Overcoming genotoxic insults are associated with reversible polyploidy. The subsequent depolyploidization results in evoking the self-renewal potential in survived cells[94].

In rodent placenta, the secondary TGC provide an example of irreversible differentiation that, however, results in formation of the low-ploidy subcellular compartments that may behave like viable cells except for renewal of their capability for proliferation. Theoretically, depolyploidization may take place in the JZ and labyrinth trophoblast cells of rat and field vole undergoing the polyploidizing mitoses, some amount of multipolar mitoses being observed here[6]. Deviation from (2^n)c in human and silver fox trophoblast[7,73,79] suggests a possibility of aneuploidy and other chromosome changes (aberrations, *etc.*) that may be even more strongly pronounced in trophoblast cells of these species. It cannot be ruled out that in human trophoblast a high degree of genomic changes also may be important for selection of cells able to survive under conditions of deep intrauterine invasion. In silver fox, a series of endoreduplication and proliferation cycles involving depolyploidization *via* multipolar mitoses and a process similar to nuclear fragmentation would also form a multifunctional system resistant to injury from the maternal tissues.

It seems to be important that different mammalian species have different programs of genome multiplication. Thus, placenta of rodents with strict spatial location of high- and low-ploidy trophoblast cells, with low incidence of deviation from (2^n)c, probably is accounted for by their strict developmental program allowing them to produce their progeny during a limited period of short pregnancy. The mammalian species with a long period of pregnancy probably stick to another strategy allowing them to generate a more specific response to stress factors that may appear occasionally during months of intrauterine development. In these cases, a

more diverse genome changes followed by selection of favorable genotypes may be useful to maintenance of placental functions.

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REFERENCES

1 **Zybina EV**, Mosjan IA. Sex chromatin bodies during endomitotic polyploidization of trophoblast cells. *Tsitologiya* 1965; **9**: 265-272
2 **Zybina EV,** Kudryavtseva MV, Kudryavtsev BN. Polyploidization and endomitosis in giant cells of rabbit trophoblast. *Cell Tiss Res* 1975; **160:** 525-537 [DOI: 10.1007/BF00225769]
3 **Nagl W**. Endopolyploidy and polyteny in differentiation and evolution. Toward an understanding of quantitative and qualitative variation of nuclear DNA in ontogeny and phylogeny. Amsterdam-N.Y.- Oxford: North-Holland publishing Company, 1978: 283
4 **Barlow PW**, Sherman MJ. Cytological studies on the organization of DNA in giant trophoblast nuclei of the mouse and rat. *Chromosoma* 1972; **47**: 119-131 [DOI: 10.1007/BF00331800]
5 **Zybina EV**, Zybina TG. Polytene chromosomes in mammalian cells. *Int Rev Cytol* 1996; **165**: 53-119 [PMID: 8900957 DOI: 10.1016/S0074-7696(08)62220-2]
6 **Zybina TG**, Zybina EV. Cell reproduction and genome multiplication in the proliferative and invasive trophoblast cell populations of mammalian placenta. *Cell Biol Int* 2005; **29**: 1071-1083 [PMID: 16316755 DOI: 10.1016/j.cellbi.2005.10.015]
7 **Zybina T**, Zybina E. Cell cycle modification in trophoblast cell population in the course of placenta formation. In: DNA replication and related cellular processes. Rijeka: InTech, 2011: 258. Available from: URL: http: //www.intechopen.com/articles/show/title/cell-cycle-modification-in-trophoblast-cell-populations-in-the-course-of-placenta-formation.
8 **Zybina EV**, Bogdanova MS, Stein GI, Vlasova TD, Zybina TG. Endopolyploidization and the interstitial invasion of the supergiant trophoblast cells of the field vole Microtus rossiaemeridionalis. *Tissue Cell* 2009; **41**: 362-366 [PMID: 19329135]
9 **Zybina TG**, Severova EL, Zybina EV, Dyban AP. A study of silver-stained nucleolus organizer regions in metaphase chromosomes of cambial cells of mouse and rat placenta. *Chromosome Res* 1997; **5**: 142-144 [PMID: 9146918 DOI: 10.1023/A:1018474309639]
10 **Zybina TG**, Zybina EV, Bogdanova MS, Stein GI. Quantitative investigation of reproduction of gonosomal condensed chromatin during trophoblast cell polyploidization and endoreduplication in the East-European field vole Microtus rossiaemeridionalis. *Reprod Biol Endocrinol* 2003; **1**: 32 [PMID: 12725646 DOI: 10.1186/1477-7827-1-32]
11 **Klisch K**, Pfarrer C, Schuler G, Hoffmann B, Leiser R. Tripolar acytokinetic mitosis and formation of feto-maternal syncytia in the bovine placentome: different modes of the generation of multinuclear cells. *Anat Embryol* (Berl) 1999; **200**: 229-237 [PMID: 10424879 DOI: 10.1007/s004290050275]
12 **Klisch K**, Bevilacqua E, Olivera LV. Mitotic polyploidization in trophoblast giant cells of the alpaca. *Cells Tissues Organs* 2005; **181**: 103-108 [PMID: 16534204 DOI: 10.1159/000091099]
13 **Wang Z**, Zhang Y, Kamer D, Lees E, Ravid K. Cyclin D3 is essential for megakaryocytopoiesis. *Blood* 1995; **86**: 3783-3788
14 **Ravid K**, Lu J, Zimmet JM, Jones MR. Roads to polyploidy: the megakaryocyte example. *J Cell Physiol* 2002; **190**: 7-20 [PMID: 11807806 DOI: 10.1002/jcp.10035]
15 **Hixon ML**, Gualberto A. Vascular smooth muscle polyploidization--from mitotic checkpoints to hypertension. *Cell Cycle* 2003; **2**: 105-110 [PMID: 12695657 DOI: 10.4161/cc.2.2.341]
16 **Gui Y**, He GH, Walsh MP, Zheng XL. Predisposition to tetraploidy in pulmonary vascular smooth muscle cells derived from the Eker rats. *Am J Physiol Lung Cell Mol Physiol* 2007; **293**: L702-L711 [PMID: 17575014 DOI: 10.1152/ajplung.00016.2007]
17 **Duncan AW**. Aneuploidy, polyploidy and ploidy reversal in the liver. *Semin Cell Dev Biol* 2013; **24**: 347-356 [PMID: 23333793 DOI: 10.1002/hep.26233]
18 **Duncan AW**, Taylor MH, Hickey RD, Hanlon Newell AE, Lenzi ML, Olson SB, Finegold MJ, Grompe M. The ploidy conveyor of mature hepatocytes as a source of genetic variation. *Nature* 2010; **467**: 707-710 [PMID: 20861837 DOI: 10.1038/nature09414]
19 **Fox DT**, Duronio RJ. Endoreplication and polyploidy: insights into development and disease. *Development* 2013; **140**: 3-12 [PMID: 23222436 DOI: 10.1242/dev.080531]
20 **Duncan AW**, Hanlon Newell AE, Bi W, Finegold MJ, Olson SB, Beaudet AL, Grompe M. Aneuploidy as a mechanism for stress-induced liver adaptation. *J Clin Invest* 2012; **122**: 3307-3315 [PMID: 22863619 DOI: 10.1172/JCI64026]
21 **Pandit SK**, Westendorp B, de Bruin A. Physiological significance of polyploidization in mammalian cells. *Trends Cell Biol* 2013; **23**: 556-566 [PMID: 23849927]
22 **Brodsky VYa**, Uryvaeva IV. Genome multiplication in growth and development. Cambridge: Univ, Press, 1985: 350
23 **Edgar BA**, Orr-Weaver TL. Endoreplication cell cycles: more for less. *Cell* 2001; **105**: 297-306 [PMID: 11348589 DOI: 10.1016/S0092-8674(01)00334-8]
24 **Chen HZ**, Ouseph MM, Li J, Pécot T, Chokshi V, Kent L, Bae S, Byrne M, Duran C, Comstock G, Trikha P, Mair M, Senapati S, Martin CK, Gandhi S, Wilson N, Liu B, Huang YW, Thompson JC, Raman S, Singh S, Leone M, Machiraju R, Huang K, Mo X, Fernandez S, Kalaszczynska I, Wolgemuth DJ, Sicinski P, Huang T, Jin V, Leone G. Canonical and atypical E2Fs regulate the mammalian endocycle. *Nat Cell Biol* 2012; **14**: 1192-1202 [PMID: 23064266 DOI: 10.1038/ncb2595]
25 **Rumyantsev PP**. Growth and hyperplasia of cardiac muscle cells. Chur, Switzerland: Harwood Acad, Publ, 1991
26 **Kudryavtsev B**, Anatskaya OV, Nilova VK, Komarov SA. Interrelation between myofibrillar and mitochondrial apparatus and cardiomyocyte polyploidy and hypertrophy in mammals. *Tsitologiya* 1997; **39**: 946-964
27 **Nagata Y**, Jones MR, Nguyen HG, McCrann DJ, St Hilaire C, Schreiber BM, Hashimoto A, Inagaki M, Earnshaw WC, Todokoro K, Ravid K. Vascular smooth muscle cell polyploidization involves changes in chromosome passenger proteins and an endomitotic cell cycle. *Exp Cell Res* 2005; **305**: 277-291 [PMID: 15817153 DOI: 10.1016/j.yexcr.2004.12.028]
28 **Nagl W**. Cdc-2 kinases, cyclins and the switch from proliferation to polyploidization. *Protoplasma* 1995; **188**: 143-150 [DOI: 10.1007/BF01280365]
29 **Therman E**, Kuhn E. Mitotic modifications and aberrations in cancer. *CRC Crit Rev Oncogenesis* 1989; **1**: 293-305
30 **Klisch K**, Thomsen PD, Dantzer V, Leiser R. Genome multiplication is a generalised phenomenon in placentomal and interplacentomal trophoblast giant cells in cattle. *Reprod Fertil Dev* 2004; **16**: 301-306 [PMID: 15304202 DOI: 10.1071/RD03101]
31 **Geitler L**. Endomitose und endomitotische Polyploidizierung. *Protoplasmatologia* 1953; **60**: 1-89
32 **Therman E**, Sarto G, Stubbliefield P. Endomitosis: a reappraisal. *Human Genetics* 1983; **63**: 13-18 [DOI: 10.1007/BF00285390]
33 **Therman E**, Sarto GE, Kuhn EM. The course of endomitosis in human cells. *Cancer Genet Cytogenet* 1986; **19**: 301-310 [DOI: 10.1016/0165-4608(86)90059-2]
34 **Hattori N**, Davies TC, Anson-Cartwright L, Cross JC. Pe-

riodic expression of the cyclin-dependent kinase inhibitor p57(Kip2) in trophoblast giant cells defines a G2-like gap phase of the endocycle. *Mol Biol Cell* 2000; **11**: 1037-1045 [PMID: 10712518 DOI: 10.1091/mbc.11.3.1037]

35 **Anisimov AP**. Endopolyploidy as a morphogenic factor of development. *Cell Biol Int* 2005; **29**: 993-1004 [DOI: 10.1016/j.cellbi.2005.10.013]

36 **Anisimov AP**. A study of genome multiplication mechanisms in the development of albumen gland cells of Succinea lauta (Gastropoda, Pulmonata). VII. Transcriptive activity of the nuclei in the endomitotic cycle. *Tsitologiya* 1997; **39**: 237-243

37 **Kuhn EM**, Therman E. The behaviour of heterochromatin in mouse and human nuclei. Cancer Genet. *Cytogenet* 1988; **34**: 143-151 [DOI: 10.1016/0165-4608(88)90181-1]

38 **Zybina TG**, Kaufmann P, Frank H-G, Freed J, Biesterfeld S. Genome multiplication of extravillous trophoblast cells in human placenta in the course of differentiation and invasion into endometrium and myometrium. II. Mechanisms of polyploidization. *Tsitologiya* 2004; **46**: 640-648

39 **Calvi BR**, Lilly MA, Spradling AC. Cell cycle control of chorion gene amplification. *Genes Dev* 1998; **12**: 734-744 [DOI: 10.1101/gad.12.5.734]

40 **Lee HO**, Davidson JM, Duronio RJ. Endoreplication: polyploidy with purpose. *Genes Dev* 2009; **23**: 2461-2477 [PMID: 19884253 DOI: 10.1101/gad.1829209]

41 **Gunderina LI**, Sherudilo AI, Mitina RL. Cycle of DNA reduplication, In polytenization of the salivary gland cells of larvae of Chironomus thummi. I. Cytophotometric and autoradiographic analysis of DNA synthesis dynamics in the individual cells of salivary glands. *Tsitologiya* 1984; **26**: 784-801

42 **Machowald AP**, Caulton JH, Edwards MK, Floyd AD. Loss of centrioles and polyploidization of follicle cells of Drosophila. *Exp Cell Res* 1979; **118**: 404-410 [DOI: 10.1016/0014-4827(79)90167-8]

43 **MacAuley A**, Cross JC, Werb Z. Reprogramming the cell cycle for endoreduplication in rodent trophoblast cells. *Mol Biol Cell* 1998; **9**: 795-807 [PMID: 9529378 DOI: 10.1091/mbc.9.4.795]

44 **Hu D**, Cross JC. Development and function of trophoblast giant cells in the rodent placenta. *Int J Dev Biol* 2010; **54**: 341-354 [PMID: 19876834 DOI: 10.1387/ijdb.082768dh]

45 **Caluwaerts S**, Vercruysse L, Luyten C, Pijnenborg R. Endovascular trophoblast invasion and associated structural changes in uterine spiral arteries of the pregnant rat. *Placenta* 2005; **26**: 574-584 [PMID: 15993707 DOI: 10.1016/j.placenta.2004.09.007]

46 **Vercruysse L**, Caluwaerts S, Luyten C, Pijnenborg R. Interstitial trophoblast invasion in the decidua and mesometrial triangle during the last third of pregnancy in the rat. *Placenta* 2006; **27**: 22-33 [PMID: 16310034 DOI: 10.1016/j.placenta.2004.11.004]

47 **Simmons DG**, Rawn S, Davies A, Hughes M, Cross JC. Spatial and temporal expression of the 23 murine Prolactin/Placental Lactogen-related genes is not associated with their position in the locus. *BMC Genomics* 2008; **9**: 352 [PMID: 18662396 DOI: 10.1186/1471-2164-9-352]

48 **Soares MJ**, Chakraborty D, Katim Rumi MA, Konno T Renaud SJ. Rat placentation: an experimental model for investigating the hemochorial maternal-fetal interface. *Placenta* 2012; **33**: 233-243 [DOI: 10.1016/j.placenta.2011.11 026]

49 **Shankar K**, Zhong Y, Kang P, Blackburn ML, Soares MJ, Badger TM, Gomez-Acevedo H. RNA-seq analysis of the functional compartments within the rat placentation site. *Endocrinology* 2012; **153**: 1999-2011 [PMID: 22355068 DOI: 10.1210/en.2011-1833]

50 **Ouseph MM**, Li J, Chen H-Z, Pecot T, Wenzel P, Thompson JC, Comstock G, Chokshi V, Byrne M, Forde B., Chong J.L. Huang K, Machiraju R, De Bruin A, Leone G. Atypical E2F repressors and activators coordinate placental development. *Dev Cell* 2012; **22**: 849-862 [DOI: 10.1016/j.devcel.2012.01.013]

51 **Sher N**, Von Stetina JR, Bell GW, Matsuura S, Ravid K, Orr-Weaver TL. Fundamental differences in endoreplication in mammals and Drosophila revealed by analysis of endocycling and endomitotic cells. *Proc Natl Acad Sci USA* 2013; **110**: 9368-9373 [PMID: 23613587 DOI: 10.1073/pnas.1304889110]

52 **Zybina TG**, Stein GI, Pozharisski KM, Zybina EV. Invasion and genome reproduction of the trophoblast cells of placenta junctional zone in the field vole, Microtus rossiaemeridionalis. *Cell Biol Int* 2014; **38**: 136-143 [PMID: 24155276 DOI: 10.1002/cbin.10187]

53 **Tamai Y,** Ishikawa T, Bösl MR, Mori M, Nozaki M, Baribault H, Oshima RG, Taketo MM. Cytokeratins 8 and 19 in the mouse placental development. *J Cell Biol* 2000; **151:** 563-572 [DOI: 10.1083/jcb.151.3.563]

54 **Hesse M**, Franz T, Tamai Y, Taketo MM, Magin TM. Targeted deletion of keratins 18 and 19 leads to trophoblast fragility and early embryonic lethality. *EMBO J* 2000; **19**: 5060-5070 [PMID: 11013209 DOI: 10.1093/emboj/19.19.5060]

55 **Jaquemar D**, Kupriyanov S, Wankell M, Avis J, Benirschke K, Baribault H, Oshima RG. Keratin 8 protection of placental barrier function. *J Cell Biol* 2003; **161**: 749-756 [PMID: 12771125 DOI: 10.1083/jcb.200210004]

56 **Gauster M**, Blaschitz A, Siwetz M, Huppertz B. Keratins in the human trophoblast. *Histol Histopathol* 2013; **28**: 817-825 [PMID: 23450430]

57 **Zybina TG**, Stein GI, Zybina EV. Endopolyploid and proliferating trophoblast cells express different patterns of intracellular cytokeratin and glycogen localization in the rat placenta. *Cell Biol Int* 2011; **35**: 649-655 [PMID: 21299496 DOI: 10.1042/CBI20100278]

58 **Enders AC**, Carter AM. Comparative placentation: some interesting modifications for histotrophic nutrition. A review. *Placenta* 2006; **27** Suppl A: S11-S16

59 **Bevilacqua E**, Hoshida M-S, Amarante-Paffaro A, Albieri-Borges A, Gomes SZ. Trophoblast phagocytic program: roles in different placental system. *Int J Dev Biol* 2010; **54**: 495-505 [DOI: 10.1387/ijdb.082761eb]

60 **Unhavaithaya Y**, Orr-Weaver TL. Polyploidization of glia in neural development links tissue growth to blood-brain barrier integrity. *Genes Dev* 2012; **26**: 31-36 [DOI: 10.1101/gad.177436.111]

61 **Ain R**, Canham LN, Soares MJ. Gestation stage-dependent intrauterine trophoblast cell invasion in the rat and mouse: novel endocrine phenotype and regulation. *Devel Dynam* 2003; **260**: 176-190

62 **Coan PM**, Conroy N, Burton GJ, Ferguson-Smith AC. Origin and characteristics of glycogen cells in the developing murine placenta. *Devel Dynam* 2006; **235**: 3280-3294 [DOI: 10.1002/dvdy.20981]

63 **Konno T**, Rempel LA, Arroyo A, Soares M. Pregnancy in the brown Norway rat: a model for investigating the genetics of placentation. *Biol Reprod* 2007; **76**: 709-718 [DOI: 10.1095/biolreprod.106.056481]

64 **Zybina EV**, Zybina TG. Modifications of nuclear envelope during differentiation and depolyploidization of rat trophoblast cells. *Micron* 2008; **39**: 593-606 [PMID: 17627829 DOI: 10.1016/j.micron.2007.05.006]

65 **Zybina EV**, Kudryavtseva MV, Kudryavtsev BN. The distribution of chromosome material during giant nucleus division by fragmentation in the trophoblast of rodents. Morphological and cytophotometrical study. *Tsitologiya* 1979; **21**: 12-20

66 **Zavarzin AA**. Synthesis and kinetics of cell populations in the mammalian onthogenesis. Leningrad: Nauka, 1967 (in Russian)

67 **Wasserlauf IE**, Ananina TV, Unger MF, Karamysheva TV, Melnikova NN, Rubtsov NB, Stegnii VN. Chromosome organization and differential banding in endomitotic nuclei of nurse cells of Calliphora erythrocephala. *Genetika* 2003; **39**:

1193-1202
68 **Zybina TG**. DNA in the nuclear fragments that appear in the course of fragmentation of the secondary giant trophoblast cells in the field vole. *Tsitologiya* 1990; **32**: 806-810
69 **Kiknadze II**, Istomina AG, Endomitosis in grasshopper. I. Nuclear morphology and synthesis of DNA and RNA in the endopolyploid cells of the inner parietal layer of the testicular follicle. *Eur J Cell Biol* 1980; **21**: 122-133
70 **Raikov IB**. The protozoan nucleus. Morphology and evolution. Wien-New York: Springer-Verlag, 1985
71 **Mossman HW**. Fetal homeostasis. The New York Academy of Sciences (NY), 1967: 50-52
72 **Leiser R**, Koob B. Development and characteristics of placentation in a carnivore, the domestic cat. *J Exp Zool* 1993; **266**: 642-656 [PMID: 8371103 DOI: 10.1002/jez.1402660612]
73 **Zybina TG**, Zybina EV, Kiknadze II, Zhelezova AI. Polyploidization in the trophoblast and uterine glandular epithelium of the endotheliochorial placenta of silver fox (Vulpes fulvus Desm.), as revealed by the DNA content. *Placenta* 2001; **22**: 490-498 [PMID: 11373160 DOI: 10.1053/plac.2001.0675]
74 **Kaufmann P**, Castellucci M. Extravillous trophoblast in the human placenta. *Trophoblast Res* 1997; **10**: 21-65
75 **Wakuda K**, Yoshida Y. DNA ploidy and proliferative characteristics of human trophoblast. *Acta Obstet Gynecol Scand* 1992; **71**: 12-16 [DOI: 10.3109/00016349209007940]
76 **Redline RW**, Hassold T, Zaragoza M. Determinants of villous trophoblastic hyperplasia in spontaneous abortions. *Mod Pathol* 1998; **11**: 762-768 [PMID: 9720505]
77 **Pötgens AJ**, Gaus G, Frank HG, Kaufmann P. Characterization of trophoblast cell isolations by a modified flow cytometry assay. *Placenta* 2001; **22**: 251-255 [PMID: 11170831 DOI: 10.1053/plac.2000.0597]
78 **Bischof P**, Campana A. Trophoblast differentiation and invasion: its significance for human embryo implantation. *Earl Pregn Biol Med* 1997; **3**: 81-95
79 **Zybina TG**, Kaufmann P, Frank H-G, Freed J, Kadyrov M, Biesterfeld S. Genome multiplication of extravillous trophoblast cells in human placenta in the course of differentiation and invasion into endometrium and myometrium. I. Dynamics of polyploidization. *Tsitologiya* 2002; **44**: 1058-1067
80 **Biron-Shental T**, Fejgin MD, Sifakis S, Liberman M, Antsaklis A, Amiel A. Endoreduplication in cervical trophoblast cells from normal pregnancies. *J Matern Fetal Neonatal Med* 2012; **25**: 2625-2628 [PMID: 22877079 DOI: 10.3109/14767058.2012.717999]
81 **Weier JF**, Weier HU, Jung CJ, Gormley M, Zhou Y, Chu LW, Genbacev O, Wright AA, Fisher SJ. Human cytotrophoblasts acquire aneuploidies as they differentiate to an invasive phenotype. *Dev Biol* 2005; **279**: 420-432 [PMID: 15733669 DOI: 10.1016/j.ydbio.2004.12.035]
82 **Anev I**, Rajasri AG, Reddy K, Pillai M. Triploidy without molar change presenting as severe pre-eclampsia and left ventricular failure at 15 weeks. *J Obstet Gynecol* 2011; **3**: 659-660 [DOI: 10.3109/01443615.2011.598966]
83 **Sarto GE**, Stubblefield PA, Therman E. Endomitosis in human trophoblast. *Hum Genet* 1982; **62**: 228-232 [DOI: 10.1007/BF00333525]
84 **Hoffman LH**, Wooding FBP. Giant and binucleate trophoblast cells in mammals. *J Exper Zool* 1993; **266**: 559-577 [DOI: 10.1002/jez.1402660607]
85 **Pfarrer C**, Hirsch P, Guillomot M, Leiser R. Interaction of integrin receptors with extracellular matrix is involved in trophoblast giant cell migration in bovine placentomes. *Placenta* 2003; **24**: 588-597 [PMID: 12828918 DOI: 10.1016/S0143-4004(03)00059-6]
86 **Zybina T**, Zybina E. Quantitative changes of nucleolar organizer in the course of polyploidization and differentiation of rat decidual cells. In: Abstracts of the … Meeting of the International Federation of Placenta Associations, Sorrento, Italy, 2001 Sept 19-23. *Placenta* 2001; **22**: A. 71
87 **Adachi S**, Minamisawa K, Okushima Y, Inagaki S, Yoshiyama K, Kondou Y, Kaminuma E, Kawashima M, Toyoda T, Matsui M, Kurihara D, Matsunaga S, Umeda M. Programmed induction of endoreduplication by DNA double-strand breaks in Arabidopsis. *Proc Natl Acad Sci USA* 2011; **108**: 10004-10009 [PMID: 21613568 DOI: 10.1073/pnas.1103584108]
88 **Mehrotra S**, Magbool SB, Kolpakas A., Murnen K., Calvi BR. Endocycling cells do not apoptose in response to DNA replication genotoxic stress. *Genes Dev* 2008; **22**: 3158-3171 [DOI: 10.1101/gad.1710208]
89 **Ullah Z**, Kohn MJ, Yagi R, Vassilev LT, DePamphilis ML. Differentiation of trophoblast stem cells into giant cells is triggered by p57/Kip2 inhibition of CDK1 activity. *Genes Dev* 2008; **22**: 3024-3036 [PMID: 18981479 DOI: 10.1101/gad.1718108]
90 **Ullah Z**, de Renty C, DePamphilis ML. Checkpoint kinase 1 prevents cell cycle exit linked to terminal cell differentiation. *Mol Cell Biol* 2011; **31**: 4129-4143 [PMID: 21791608]
91 **Kudryavtsev BN**, Kudryavtseva MV, Sakuta GA, Stein GI. Human hepatocyte polyploidization kinetics in the course of life cycle. *Virchows Arch B Cell Pathol Incl Mol Pathol* 1993; **64**: 387-393 [PMID: 8148960 DOI: 10.1007/BF02915139]
92 **Erenpreisa J**, Cragg MS. MOS, aneuploidy and the ploidy cycle of cancer cells. *Oncogene* 2010; **29**: 5447-5451 [DOI: 10.1038/onc.2010.310.20676137]
93 **Salmina K**, Jankevics E, Huna A, Perminov D, Radovica I, Klymenko T, Ivanov A, Jascenko E, Scherthan H, Cragg M, Erenpreisa J. Up-regulation of the embryonic self-renewal network through reversible polyploidy in irradiated p53-mutant tumour cells. *Exp Cell Res* 2010; **316**: 2099-2112 [PMID: 20457152 DOI: 10.1016/j.yexcr.2010.04.030]
94 **Erenpreisa J**, Cragg MS. Three steps to the immortality of cancer cells: senescence, polyploidy and self-renewal. *Cancer Cell Int* 2013; **13**: 92 [PMID: 24025698 DOI: 10.1186/1475-2867-13-92]

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REVIEW

Role of SOX2 in foregut development in relation to congenital abnormalities

Kim Schilders, Joshua K Ochieng, Cornelis P van de Ven, Cristina Gontan, Dick Tibboel, Robbert J Rottier

Kim Schilders, Joshua K Ochieng, Cornelis P van de Ven, Cristina Gontan, Dick Tibboel, Robbert J Rottier, Departments of Pediatric Surgery of The Erasmus MC-Sophia Children's Hospital, 3000 CA Rotterdam, The Netherlands
Cristina Gontan, The Department of Reproduction and Development of the Erasmus MC, 3015 CN Rotterdam, The Netherlands
Robbert J Rottier, Cell Biology of the Erasmus MC, 3000 CA Rotterdam, The Netherlands
Author contributions: Schilders K, Ochieng JK, Gontan C and Rottier RJ wrote the first draft of the manuscript; van de Ven CP and Tibboel D added all patient information; Schilders K, Tibboel D and Rottier RJ provided critical input and comments; all authors edited the manuscript.
Correspondence to: Robbert J Rottier, PhD, Departments of Pediatric Surgery of The Erasmus MC-Sophia Children's Hospital, PO Box 2040, 3000 CA Rotterdam,
The Netherlands. r.rottier@erasmusmc.nl
Telephone: +31-10-7044140 Fax: +31-10-7044468
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Abstract

The uptake of the two essential ingredients for life, oxygen and nutrients, occurs primarily through the oral cavity, but these two lifelines need to be separated with high accuracy once inside the body. The two systems, the gas exchange pulmonary system and the gastro-intestinal feeding system, are derived from the same primitive embryonic structure during development, the foregut, which need to be separated before birth. In certain newborns, this separation occurs not or insufficiently, leading to life threatening conditions, sometimes incompatible with life. The development of the foregut, trachea and lungs is influenced and coordinated by a multitude of signaling cascades and transcription factors. In this review, we will highlight the development of the foregut and pulmonary system and focus on associated congenital abnormalities in light of known genetic alterations with specific attention to the transcription factor SOX2.

Key words: SOX2; Congenital foregut abnormalities; Lung development

Core tip: Foregut abnormalities are complicated congenital diseases which still lack knowledge of the origin. This review highlights foregut development and associated abnormalities, specifically focussing on the transcription factor SOX2.

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FOREGUT DEVELOPMENT

Gastrulation is the process that adds complexity to the developing organism and results in a triploblastic animal by formation of the three germ layers, ectoderm, mesoderm and endoderm. At embryonic day 8 (E8.0) in mice (comparative to 3 wk in human), the sheet of endodermal cells starts to invaginates ventrally at the anterior and posterior intestinal portals, which subsequently migrate towards each other to form the primitive gut from the future mouth to anus[1]. At E9.0, the notochord delaminates from the dorsal endoderm and will eventually be situated between the primitive gut and the neural tube. The notochord serves in this phase of development as a strong signaling center, secreting morphogens like Sonic hedgehog (Shh) to pattern the endoderm as well as the neural tube[2]. Another signaling center associated with the early patterning and morphogenesis of the foregut is the heart mesoderm, which secretes Fibroblast growth factors (Fgf). High levels of Fgf signals activate lung specific genes while lower levels of Fgf activate liver specific

Table 1 Gestational ages in human and mouse during the five stages of lung development[8]

Phases of lung development	Gestational age	
	Human	Mouse
Embryonic phase	Weeks 3-7	E9-11.5
Pseudoglandular phase	Weeks 5-17	E11.5-16.6
Canalicular phase	Weeks 16-25	E16.6-17.4
Saccular phase	Weeks 24-38	E17.4-PN5
Alveolar phase	Weeks 36 to maturity	PN5-30

E: Embryonic age; PN: Post natal age.

genes[3]. The prospective lung field, the area which will eventually lead to the emergence of the primitive lung bud, is subsequently patterned by retinoic acid (RA) signaling. The RA receptor (RARα) is required to maintain RA signaling and to assist the effects of RARβ, which induces the expression of Fgf10. RA signaling integrates the Wnt and transforming growth factor beta (Tgfβ) pathways by inhibiting the expression of the Wnt antagonist Dickkopf-1 and by preventing the expression of Tgfβ[4,5]. Overall, the foregut is regionalized as shown by the various dorsal-ventral gradients of morphogens and subsequent transcription factors. This pattern of expression is essential for proper development of the trachea, esophagus and lungs, and disturbances in these patterns result in various trachea-lung defects (see below).

Lung development

The lung primordium arises from the ventral foregut as a primary bud, just anterior to the developing stomach around embryonic day 9.5 in mice or week 4 in humans[6,7]. The lung bud splits in two buds, the future left and right bronchus, elongates and the proximal part separates into oesophagus and trachea, while distally the bronchial tree is formed through a process called branching morphogenesis[6].

Development of the lung can be divided into five distinct, but overlapping phases based on morphology (Table 1)[8]. During the earliest phase, the embryonic phase, the lung buds are formed from the primitive foregut, the mayor bronchi are formed and the tracheal-esophageal tube is dividing. Several signaling cascades direct the early embryonic morphogenetic events and cell fate decisions including Tgfβ, Bone Morphogenetic Proteins (BMPs), Shh, Wnt, and Fgf families, which will be discussed in more detail[9,10]. As development of the lung advances, the embryonic endoderm undergoes progressive fate decisions that generate epithelial progenitor cells with increasingly restricted developmental potential over time. The next phase, the pseudoglandular phase is characterized by the commencement of differentiation of epithelial cells. Also, the bronchial tree and all terminal bronchioles are formed. The pseudoglandular phase is followed by the canalicular phase and the saccular stage[6]. During the canalicular phase, the conducting airways are completed and the respiratory portions of the lung as well as the capillary bed are formed, while during the saccular phase the terminal tubes narrow, giving rise to small saccules and the endoderm begins to differentiate into specialized alveolar type I and type Ⅱ cells[6]. The last phase, the alveolar phase, is characterized by the establishment of secondary septa resulting into alveolar formation, which mainly takes places after birth[8,11].

Regulation of foregut and lung development

The morphogenesis of the foregut and lung is subsequently regulated by a myriad of transcription factors and signaling cascades. The molecular and cellular events contributing to lung development and the separation of the trachea and esophagus have been extensively described in recent reviews[6,10,12]. Regionalization of the different parts of the gut is controlled by the localized expression of Homeobox (*Hox*) genes[13]. *Hoxa3* and *Hoxb4* are expressed in the foregut endoderm, whereas *Hoxc5* and *Hoxa13* are expressed in the midgut and hindgut endoderm, respectively[13,14]. During tracheal-eosophageal development, Shh is specifically and dynamically expressed during the patterning of the ventral foregut whereas its expression is transiently expressed in the tracheal endoderm[15]. During the early stages of branching morphogenesis, Shh is expressed in the epithelium, with the highest levels of expression in the tips. Later, there is downregulation of Shh in the proximal parts of the airways while distally the expression sustains[16].

SOX GENES AND FOREGUT DEVELOPMENT

Transcription factors that show specific expression profiles in the endoderm, include members of the SRY-related High-Mobility Group (HMG) transcription factors[17,18]. The Sex-determining region on the Y chromosome (*Sry*) gene, was the first identified member of the SOX family of transcription factors[19,20]. SOX family members are highly conserved across species and they were originally identified by homology, as they contain an HMG box closely related to that of the *Sry* gene[21]. Therefore, Sry gave the *SOX* gene family its name; Sry-related HMG box, hence "SOX", followed by a number corresponding to the order of discovery[19]. SOX proteins have properties of both classical transcription factors and architectural proteins[22]. They function as classical transcription factors, either activating or repressing specific target genes through interaction with different partner proteins.

All SOX factors bind DNA *via* their HMG domain and recognize the same consensus motif 5'-(A/T)(A/T)CAA(A/T)G-3'[23]. The transcriptional function of SOX proteins dependent on the cell type and the promoter context, and they often have functional redundancy among each other[22]. In contrast to other transcription factors which mainly target the major groove, SOX proteins interact with the minor groove of the DNA helix and, as a consequence, induce a sharp bend in the DNA[22]. The DNA bending capacity of SOX proteins can be functionally important for several reasons. It may

Table 2 The role of *SOX* genes in diseases

SOX gene	Chromosome location	Disease
SOX2	3q26.3-q27	Microphthalmia, syndromic 3 optic nerve hypoplasia, abnormalities of the central nervous system, CHARGE-syndrome[65], AEG-syndrome[57], EA/TEF[58], CPAM[39,40]
SOX3	Xq27.1	Mental retardation, X-linked with isolated growth hormone deficiency, infundibular hypoplasia, hypopituitarism[117]
SOX9	17q23	Campomelic dysplasia with autonomic XY sex reversal[117], Pierre-Robin syndrome[118]
SOX10	22q13.1	Waardenburg-Shah syndrome, Yemenite deaf-blind hypopigmentation syndrome, peripheral demyelinating neuropathy, central dysmyelinating leukodystrophy, Waardenburgh syndrome, Hirschprung's disease[117]
SOX11	2p25	Unknown
SOX17	8q11.23	Unknown
SOX18	20q13.33	Hypotrichosis-lymphedema-telangiectasia syndrome[117]

EA/TEF: Esophageal atresia/tracheoesophageal fistula; CPAM: Congenital pulmonary and airway malformations.

bring different regulatory regions of the target gene into close proximity. Thereby, it facilitates the formation of enhanceosomes, *i.e.*, functionally active complexes of transcription factors on different gene enhancer sequences[24]. It also allows the interaction of distant enhancer nucleoprotein complexes with the basal transcription machinery[25,26]. The local changes in chromatin structure induced by SOX proteins may facilitate the recruitment of higher-order architectural factors (like polycomb or trithorax protein groups)[27]. Bending of DNA by SOX proteins could also act in a negative way by preventing the binding of other factors to adjacent sites in the major groove[27].

SOX proteins have been identified in all animal species (birds, reptiles, amphibians, fish, insects, and nematodes)[28] and mutations in several of the *SOX* genes have been implicated in the pathogenesis of human congenital anomalies and syndromes (Table 2).

SOX genes are expressed in diverse and dynamic patterns during embryogenesis. During development members of the SOX family are expressed in almost every tissue of the embryo, and also in a number of adult tissues[29,30]. The expression of a specific SOX transcription factor is not necessarily restricted to a particular cell type or lineage. Their expression pattern during development appears to correlate with early cell fate decisions. For example, Sry is expressed in the undifferentiated male gonad and is quickly down regulated once the decision is made to initiate male development[31-33].

To date, four members from the *SOX* gene family are known to be involved in lung organogenesis, *SOX2*, *SOX9*, *SOX11* and *SOX17*[8,34-39]. *SOX9* was found throughout lung morphogenesis as a downstream gene of Shh and modulated by BMP4 and Noggin. Using epithelial specific gain and loss function mouse models, SOX9 has been shown to play a crucial role in branching morphogenesis through controlling a balance between proliferation and differentiation[40]. In another study, knock out of SOX9 in the mesenchyme demonstrated that it plays a crucial role in differentiation of the lung tracheal epithelium[41]. SOX9 is required for formation and patterning of tracheal cartilage by a mechanism mediated by Fgf18[42,43]. SOX9 promotes proper branching morphogenesis by controlling the balance between proliferation and differentiation and regulating the extracellular matrix and can be used as a marker for the distal epithelium[40]. SOX11 has been suggested to be involved in development and plays a key function in tissue remodeling, including the lung[35]. SOX11 deficient mice die immediately after birth because of significant lung hypoplasia and other tissue defects[35]. SOX17 was shown to be crucial early after gastrulation for the formation of definitive endoderm, which gives rise to the lung, liver, pancreas, stomach, and gastrointestinal tract[44]. In the lung, SOX17 is expressed in the respiratory epithelial cells at embryonic day 18 in mice and becomes primarily restricted to ciliated cell in the postnatal and adult lung[36]. Ectopic expression of SOX17 in lung epithelial cells inhibits peripheral epithelial cell differentiation and results in the activation of the cell cycle and the initiation of progenitor-like cell behavior in mature lung cells[36]. SOX17 has been shown to impair the expression of Tgfβ1 responsive inhibitors, p15, p21 and p57, while inhibiting Tgfβ1 and Smad3 transcriptional activity[36].

SOX2

The mouse *SOX2* gene has been mapped to chromosome 3[45]. SOX2 plays crucial roles during different stages of vertebrate embryonic development and its expression is temporally and spatially regulated[46]. SOX2 expression starts at the morula-stage of embryo development. In blastocysts it is specifically detected within the cells of the inner cell mass (ICM). Expression continues in the epiblast, the tissue that will give rise to the embryo and germ cells[47]. SOX2 is also expressed in embryonic stem cells, which are derived from the ICM. During early gastrulation, SOX2 expression in the embryo is restricted to the anterior ectoderm, which gives rise to neuroectoderm and anterior surface ectoderm, while the extraembryonic expression becomes confined to the chorion[48,49]. At later stages of embryonic development, SOX2 is expressed in the brain, neural tube, eyes, sensory placodes, branchial arches, gut endoderm, and the germ cells[47,50-55]. When the arches develop, SOX2 continues to be expressed in the primitive foregut endoderm. Later, SOX2 is present in the epithelium of foregut-derived organs, including the tongue, esophagus, trachea, proximal lung and stomach[39,47,50,56,57].

The lack of SOX2 expression in mice results in early embryonic lethality[47]. *SOX2* null mutant mouse embryos implant but fail to develop an egg cylinder or epiblast, and they die before gastrulation because SOX2 is required in the ICM of the blastocyst[47]. Other mutations that only affect SOX2 regulatory elements can cause deafness, defects in the inner ear, circling behavior, and a yellow coat color[45]. The use of two *SOX2* hypomorphic mutants showed a dose-dependent role of SOX2 in the development of the retina and the differentiation of the foregut endoderm[54,58]. Heterozygous mutations in *SOX2* have been associated in human with severe structural malformations of the eye, bilateral anophthalmia (absent eye) and microphthalmia (small eye), and anophthalmia-esophageal-genital (AEG) syndrome[57]. In AEG infants the esophagus and trachea fail to separate normally and the trachea is connected to the stomach by an abnormal distal esophagus[57,59,60]. These symptoms underwrite the developmental functions for SOX2, as found in SOX2 hypomorphic mice described above.

SOX2 in foregut and lung development: SOX2 is expressed throughout the early foregut epithelium, but becomes restricted to the dorsal epithelial cells at embryonic day 9.5, whereas Nkx2.1 is reciprocally expressed in the ventral epithelium[58,61]. SOX2 is expressed in the epithelial cells of the foregut at E9.5. From E11.5 until E14.5, SOX2 is exclusively expressed in the epithelial cells of the non-branching developing airways and it remains expressed in the epithelial cells of the conducting airways after birth. So SOX2 is exclusively expressed at the non-branching airways[39].

Previously, it was shown that ectopic expression of SOX2 in epithelial cells of the lung result in abnormal alveolar formation, enlarged airspaces and a decrease in the number of airways, indicating that SOX2 modulates branching morphogenesis. Also, an increased number of neuroepithelial cells and (pre-) basal cells was observed. This indicates that SOX2 is important in cell fate choice and epithelial differentiation[39]. More recently it was shown that SOX2 regulates the emergence of lung basal cells by directly activating the transcription of the basal cell master gene Trp63, and the emergence of bronchioalveolar stem cells[62].

The proper dorsal-ventral patterning of SOX2 and Nkx2.1 is critical for foregut morphogenesis. Down-regulation of SOX2 leads to the formation of esophageal atresia/tracheoesophageal fistula (EA/TEF) in SOX2 hypomorphic mutants[58], whereas deletion of Nkx2.1 leads to defects in foregut separation and the formation of EA/TEF associated with high SOX2 expression in the epithelium[58,63]. Similarly, the epithelial cells in the fistula of SOX2 hypomorphic mutants express high levels of Nkx2.1 suggesting that low level of SOX2 is required for Nkx2.1 expression to expand dorsally and reprogram the dorsal epithelium to a respiratory fate[58]. These findings suggested that the dorsal-ventral arrangement of SOX2 and Nkx2.1 is essential for foregut separation and the subsequent differentiation of epithelial progenitor cells into oesophageal and tracheal epithelium and lung buds[12].

Using Chromatin Immuno Precipitation it was shown that SOX2 directly binds to the promoter region of the *NKX2.1* gene in human embryonic stem cells and this binding resulted in the inhibiton of NKX2.1 transcription[64]. Other interesting SOX2 target genes that are involved in early lung morphogenesis are members of the Notch (JAG1) pathway and Shh pathway (GLI2, GLI3)[65]. Since the activity of SOX2 depends on its interaction with other proteins it is of high importance to reveal its interacting partners. Recently some of these partners were identified in embryonic and neural stem cells[65,66]. One of the partners identified is Chromodomain-Helicase-DNA-Binding Protein7 (CHD7), which plays a major role in CHARGE syndrome. As mentioned before, SOX2 plays a role in AEG syndrome which shows many similarities with CHARGE syndrome. SOX2 and CHD7 also regulate common target genes, like *MYCN*, *JAG1* and *GLI2/3*. These genes are involved in syndromes that are characterized by the same malformations as AEG and CHARGE syndromes. Gene networks like this *SOX2-CHD7*-regulated network can be used to better understand the molecular basis of various human diseases and therefore associating partners in the lung epithelium could help us to reveal the mechanisms underlying lung-related abnormalities[65].

FGF SIGNALING

Another study using *in vitro* organ cultures demonstrated that Fgf10 signaling inhibits SOX2 expression in the mouse foregut[58]. Mesenchymal expression of Fgf10 around the distal ends of the lung epithelium functions as a chemoattractant by binding to the epithelial expressed Fgf receptor 2b (Fgfr2b) leading to branching and outgrowth of the epithelium[67,68]. The functional interaction between Fgf10 and Fgfr2b was shown by the high similarity between the Fgf10-null and Fgfr2b-null mouse mutants[3,69]. Fgf10 knockout mice developed normal trachea, but completely lacked lung structures[69,70], whereas targeted deletion of Fgfr2b prevented branching, causing the trachea to terminate as a blind-ended sac[71]. Conditional gene inactivation studies further demonstrate that both Fgf10 and Fgfr2b are required for a normal branching program and proper proximal-distal patterning of the lung[72]. Recently, it was shown that ubiquitous overexpression of Fgf10 throughout the lung could rescue lung agenesis in Fgf10 knockout mice, suggesting that precise localization of Fgf10 expression is not required for lung branching morphogenesis. Rather, Fgf10 signaling prevents cells from expressing SOX2 by the activation of β-catenin. As the lung bud grows, the cells become more distant from the Fgf10 source and start to adopt a more proximal cell fate expressing SOX2. When SOX2 is ectopically expressed in the distal epithelial cells of the developing airways, these cells are no longer responsive to

Fgf10 and differentiate into proximal cells, which results in reduced branching and formation of cyst-like structures[39,62].

WNT SIGNALLING

Receptor tyrosine kinases (RTKs), like the Fgfr, are able to activate Wnt/β-catenin through the Erk/MAPK mediated phosphorylation of the Wnt co-receptor Lrp6 on Ser1490 and Thr1572, leading to an increased cellular response to Wnt. Moreover, RTKs directly phosphorylate β-catenin on the Tyr142 residue, which causes its release from membrane bound cadherin complexes[73]. In turn, Fgfr2b expression is induced by activation of epithelial β-catenin activation, which results in an increase of Fgf10 signaling[74]. This regulation of distal epithelial progenitors by β-catenin suggests the progressive signaling cascade where Fgf10 regulates branching morphogenesis *via* Wnt signaling. Epithelial specific expression of Wntless, a cargo receptor protein important for directing Wnt ligands, has recently been shown to be important for lung differentiation and vasculature development probably by modulating the secretion of Wnt ligands[75].

At embryonic day 9.5, Wnt signaling is active in the ventral side of the unseparated foregut tube, where the Wnt ligands Wnt2 and Wnt2b are highly expressed[61,76]. Wnt2 and Wnt2b are secreted by mesenchymal cells of the ventral foregut and signal through the canonical β-catenin pathway to specify lung progenitors in the foregut endoderm[61,76]. Conditional inactivation of β-catenin in the foregut endoderm results in the absence of both trachea and lung, whereas expression of a constitutively active β-catenin mutant results in the expansion of the earliest respiratory marker, Nkx2.1, and a loss of the SOX2 positive domain[61]. Later in development, Wnt/β-catenin signaling is required for proper proximal-distal patterning of the lung[74]. Wnt7b is expressed in the endoderm of the early foregut and its deletion does not disrupt foregut separation, but results in irregular lung branching morphogenesis and vasculature development[77]. Mesenchymal Wnt2 and epithelial Wnt7b cooperate with Pdgf signaling to promote mesenchymal differentiation[78].

Respiratory endodermal specific expression of a constitutive active β-catenin isoform showed that canonical Wnt signaling is not required for the development of alveolar epithelium[79,80]. However, the formation of proximal epithelium was impaired, because ectopic Wnt signaling induced the expression of Tcf1 and Lef1 at the expense of SOX2 and Trp63[79]. On the other hand, conditional deletion of β-catenin in respiratory epithelium resulted in the loss of alveolar structures. Selective loss of bronchiolar lineages with continued proliferation may result in cystic lesions of the lung resembling an anomaly known in humans as congenital pulmonary and airway malformations (CPAM)[80].

Ectopic expression of Wnt5a in the respiratory epithelium resulted in increased Fgf10 expression and a reduction in epithelial Shh expression[81]. The precise dose and timing of Fgf and Wnt signaling lead to the induction of Shh expression in the respiratory epithelium[3,76]. The paracrine effect of Shh on the surrounding mesenchyme results in the Foxf1 and Gli1/Gli3 mediated expression of BMP4.

BMP SIGNALING

BMP signaling plays prominent roles in foregut separation and lung development, however the molecular mechanisms controlling temporal-spatial BMP signaling dynamics in foregut organogenesis are poorly understood[82]. In the unseparated foregut tube, BMP4 is expressed in the ventral mesenchyme, while BMP7 and the BMP antagonist Noggin are enriched in the dorsal endoderm[83]. Ablation of Noggin resulted in increased BMP signaling in the foregut and the formation of EA/TEF[84]. These embryos showed abnormal delamination of the notochord from the early definite endoderm epithelial sheet, resulting in epithelial cells of endodermal origin being present in the notochord[84]. Subsequent deletion of either BMP4 or BMP7 in these Noggin null mice rescued the separation defects[83,84]. Recently, it was shown that Noggin is required to attenuate BMP signaling in order to allow the notochord to delaminate form the dorsal foregut endoderm[85]. Tissue specific ablation of BMP4 in the early foregut endoderm resulted in tracheal agenesis accompanied by reduced cellular proliferation in the epithelial and mesenchymal compartments. However, the trachea does not separate from the foregut and Nkx2.1 expression is conserved in the ventral endodermal epithelium, suggesting that BMP4-mediated signaling is essential for separation but not for the initial specification of the tracheal epithelium[86]. Similarly, conditional inactivation of BMPr1a and BMPr1b in the foregut leads to tracheal agenesis, a decrease of Nkx2.1 expression and a ventral expansion of SOX2 and Trp63 expression. Subsequent activation of Wnt signaling did not promote respiratory differentiation. Deletion of SOX2 in the BMPr1a/b deficient mouse rescued the foregut separation defect, showing that SOX2 is downstream of BMP signaling[87]. Ectopic expression of SOX2 in the distal lung buds showed that Fgf-Erk signaling was abrogated at the expense of BMP-Smad signaling[39].

RELATIONSHIP BETWEEN SOX2 AND CONGENITAL DEFECTS OF THE FOREGUT

Congenital malformations of the lung constitute a spectrum of lesions that originate during the embryonic period. Incidences of congenital defects of the foregut are in the range of 1:11.000-35.000 pregnancies (World Health Organization). Patients present a broad range of clinical manifestations ranging from intra uterine death to significant illnesses at birth with a variable severity of

respiratory symptoms and later on distress and repeated chest infections. However a number, although impressive at repeated prenatal ultrasound or magnetic resonance imaging may remain asymptomatic for long periods and significantly regress in the course of pregnancy. Congenital defects of the foregut occur either in isolated cases or as part of a complex syndrome[88]. The causes of most of these malformations as well as their molecular genetic background are still unknown. Different types of congenital lung malformations can be distinguished, which will be briefly discussed.

CPAM

CPAM constitute a spectrum of lesions that originate during the embryonic period. The prevalence of congenital lung malformations has seemingly increased over the last decade probably due to better antenatal ultrasound screening and is estimated at 1 in 3000 pregnancies[89]. Although most newborns with antenatally diagnosed congenital lung malformations are asymptomatic at birth, approximately 10% show respiratory insufficiency. Secondary infections of these lesions occur in approximately 5% of unoperated children.

The different types of congenital pulmonary and airway malformations are classified in bronchopulmonary malformations, pulmonary hyperplasia, congenital lobar overinflation and other cystic lesions[90].

Bronchogenic cyst: A bronchogenic cyst is often a solitary cyst in the mediastinum or in the lung parenchyma filled with fluid. Their structural lining resembles that of the bronchus, cartilage and bronchial-type glands included. Symptoms at birth are mostly due to compression of surrounding structures, especially bronchial structures resulting in hyperinflation of lung parenchyma distal to the obstruction. Symptoms at later age are mainly due to infection. Etiology is probably similar to other duplication cysts as aberrant bud formation from the foregut structures. The molecular biology has not been studied so far.

Bronchial atresia: Bronchial atresia, mostly asymptomatic often results in overinflation of a lobe, segment or even smaller part of the lung depending on the level of bronchus being atretic. Symptoms are rare. Etiology is unknown and may be similar to the reasons of bronchial blockage in congenital lobar overinflation.

Cystic adenomatoid malformation stocker type 1 and type 2: Although congenital cystic adenomatoid malformation (CCAM) pathogenesis is unknown, several authors have hypothesized that different types of CCAM originate at different stages of lung development. Abnormal airway development during branching morphogenesis probably results in specific areas of the lung where terminal bronchioles overgrow and alveolar formations are absent[88]. Another hypothesis postulates that CCAM originate as a result of imbalance between cell proliferation and apoptosis during airway branching[62,91,92]. Type 1 CCAM consists of a few large cysts with bronchiolar configuration and a lining of respiratory epithelium overlying fibroelastic tissue and small amounts of smooth muscles. It may have a systemic arterial supply. Type 2 CCAM are multicystic lesions (cysts < 2 cm) often localized in one lobe, although multiple lobes can be affected.

Aberrant expression of genes involved in lung development has been shown to result in CCAM-like phenotypes. Transiently induced overexpression of SOX2[39,62], Fgf10[93], orthotopic overexpression of Fgf9 and heterotopic overexpression of Fgf7 show perturbations of lung morphogenesis some mimicking CCAM type 1 and 2 depending on the time of overexpression.

In human resection specimens increased levels of the transcription factors HOXB5, TTF1, Fgf9[94,95] as well as changed expression patterns of the adhesion molecules α-2 integrins and E-cadherin, increased levels of Clara cell marker CC-10 and reduced expression of Fatty acid binding protein-7 have been described[96]. Moreover, microarray data revealed a 6 fold up-regulation of SOX2 in CCAM tissue compared with controls[96]. These findings correspond with recently published data, describing the generation of CCAM-like phenotype by overexpressing SOX2 in mouse. In a comparative study, expression of SOX2 in human CCAM tissue was identified[62]. Different etiology for type 1 and type 2 may be supported by recent findings that SOX2 is expressed in epithelial lining of cystic lesions in CCAM type 2, but not in CCAM type 1. Moreover, TRP63 was co-expression in SOX2 positive cells, suggesting that the epithelium had proximal characteristics (Ochieng *et al*[62], 2014).

Extralobar sequestration: Extralobar sequestration (ELS) are characterized by normal, non-functioning lung tissue without connection with the bronchial tree and often receive blood supply from the systemic circulation. Mainly found in the left lower chest, these lesions can also be found in or below the diaphragm. In contrast to CCAM, associated anomalies like vertebral and chest wall deformities and congenital heart disease are described. In 5%-15% of patients with congenital diaphragmatic hernia an ELS is found at operation. Although often asymptomatic, antenatal diagnosis can be very helpful to detect congestive heart failure caused by the arteriovenous shunting through the anomalous systemic blood supply. Late symptoms are mainly infectious. One of the genes that is thought to be involved in the pathogenesis of this anomaly is the HOXB5 gene. It is previously shown that this gene is involved in airway branching[97,98].

Pulmonary hyperplasia and related lesions

Laryngeal atresia: Laryngeal atresia causes congenital high airway obstruction syndrome (CHAOS) at birth in the absence of a tracheoesophageal fistula. Prenatally, a polyhydramnios, large lung volume and inverted diaphragm are associated with fetal hydrops. Survivors are only described in those patients who have a tracheo-

esophageal fistula or a pinpoint laryngeal connection to relieve pressure from the lungs. Still, these patients may suffer from tracheobronchomalacia and diaphragmatic dysfunction due to increased lung extension during pregnancy. CHAOS can be associated with Fraser syndrome[99]. If a bigger tracheoesophageal connection exists, prenatal diagnosis is difficult and diagnosis is only made at birth due to severe dyspnea. As a cause of laryngeal atresia, failure of recanalization of the laryngeal membrane is described. No detailed molecular analysis of lungs of laryngeal atresia, either pre- or postnatally, has been reported.

Solid or cystic adenomatoid malformation, stocker type 3: The type 3 lesion, which accounts for 5%-10% of cases, occurs almost exclusively in males, and is associated with maternal polyhydramnios in nearly 80% of the cases. These are large, non-cystic bulky lesions, compressing the adjacent lung and mediastinum. Microscopically, randomly scattered bronchiolar/alveolar duct-like structures are lined by low cuboidal epithelium and surrounded by "alveoli" also lined by cuboidal epithelium. The virtual absence of any small, medium, or large pulmonary arteries in this type of lesion is remarkable.

Congenital lobar overinflation

Congenital Iobar overinflation, or Congenital lobar emphysema (CLE), is a rare lung malformation with an incidence ranging from 1:20000 births to 1:30000 births[100-102]. CLE is characterised by distended alveoli distal to the terminal bronchiole with destruction of the lining of the lobes, in contrast to a polyalveolar lobe where the number of alveoli is increased. CLE usually affects the left upper or right middle lobe[103]. Prenatal diagnosis can be made when a hyperechogenic lung is seen, but discrimination with CCAM and ELS is difficult at that point. Although respiratory failure can be present at birth due to compression of normal lung and displacement of the heart, many patients are asymptomatic. As a cause of CLE, a disruption of normal bronchopulmonary tree development is described with dysplastic cartilage, mucosal overgrowth, main stem bronchial atresia or external compression from abnormal cardiovascular structures. No specific genetic anomalies have been linked to this anomaly so far.

Bochdalek type of Congenital diaphragmatic hernia

Bochdalek type of Congenital diaphragmatic hernia (CDH) is characterized by a posterolateral defect mostly in the left diaphragm, which results in herniation of the abdominal organs into the chest[104,105]. Subsequent pulmonary hypoplasia and pulmonary hypertension cause severe respiratory failure at birth. Lung hypoplasia is characterized by reduced alveolar air spaces lacking secondary septae, thickened alveolar walls and increased interstitial tissue. In the pulmonary vessels hyperplasia of the median and increased adventitial layer of the arterial wall is well described. The incidence of CDH is approximately one in 2500 births and the underlying cause of CDH is still unknown in a large number of patients.

Several links to gene loci have been found partly based on animal experiments[106] and several members of the vitamin A-RA pathway have been implicated in the occurrence of CDH, such as vitamin A deficiency, STRA6 and RALDH2[104,105,107]. Moreover, some genes that are downstream of this pathway, like COUP-TFⅡ, FOG2, GATA4 and GATA6, have also been found to be associated with CDH review[107,108]. Recent exome sequencing identified a novel candidate gene, PIGN, aside from the known FOG2 involvement[109,110]. A direct link of CDH and SOX2 has not been described.

Esophageal atresia

Esophageal atresia with or without TEF has an incidence ranging between one in 2500 to one in 4500 births[111]. In EA, the proximal esophagus is blunt-ended, while the distal part is connected to the trachea. This connection and the position of the atresia varies between patients and leads to the classification of five subtypes[112]. In 85% of cases the esophageal atresia (EA) is accompanied by a distal tracheoesophageal fistula. Approximately half of the patients suffer from associated anomalies, as recently been reviewed[113].

The genetics of EA/TEF is complex, and several studies have indicated putative factors associated with either the multifactorial syndromes, or with EA/TEF. Based on murine models of this anomaly, several candidate genes and pathways have been identified, such as the receptors of the RARα/RARβ, members of the SHH-PTC-GLI pathway (Shh, Gli2/3, Foxf1), BMP signaling (Noggin) and some transcription factors (Hoxc4, Ttf-1, Pcsk5, Tbx4, SOX2)[113].

Some of these candidate genes seem to be associated with human EA/TEF, such as FOXF1, PCSK5, SHH, NOG and SOX2. Moreover, other human genes have been associated with EA/TEF as part of several syndromes, such as Feingold (MYCN), Opitz G (MID1), Fanconi anemia (FANCA/C/D/G) and CHARGE (CHD7). Recently, CHD7 was shown to directly associate with SOX2, thereby linking CHARGE syndrome with AEG[65]. Moreover, it was shown that these two proteins activated the transcription of a number of genes that are implicated in related syndromes, like *Shh*, *Gli2/3*, *Mycn*.

Alveolar capillary dysplasia

Although alveolar capillary dysplasia with misalignment of pulmonary veins (ACD/MPV) is a very rare condition without known incidence it has a dismal prognosis. The diagnosis is most likely underreported because it can only be made by histological examination of lung tissue. Newborn patients present with respiratory failure, hypoxemia, metabolic acidosis, pulmonary hypertension and right ventricular failure. Chest X-ray can be interpreted as normal but might show diffuse haziness or ground-glass opacities. Associated congenital anomalies may be present, especially of the genitourinary, gastrointestinal

and cardiovascular system. Histologically, a decreased number of pulmonary capillaries is observed, distantly from the alveolar epithelium and thickened septae with a malposition of pulmonary veins close to pulmonary arteries. Often lymphangiectases are seen. Pulmonary arteries show medial hypertrophy with muscularization of distal arterioles. Although treatment response especially to therapy relieving pulmonary hypertension has been described, effects are transient. Late presenters and long-term survivors very rarely have been described and might be due to a lesser degree of histological changes.

Deletions in chromosomal region 16q24.1q24.2 have been described. The smallest region of overlap in these deletions contains the FOX transcription factor gene cluster, including *FOXF1*, *FOXC2* and *FOXL1*. Findings in the mouse model with heterozygous deficiency of FOXF1 are similar to those in humans with ACD/MPV whereas mouse embryos with homozygous deficiency die at E9,5 due to pulmonary vascular abnormalities. A relationship with SOX2 never has been described[114-116].

CONCLUSION

SOX2 has only been found to be associated with a limited number of specific subsets of congenital anomalies. Modulating the expression levels of SOX2 during trachea and lung development in mice have led to abnormalities which relate to human conditions, such as CPAM and EA/TEF[39,58,62]. Interestingly, CHD7, which is linked to CHARGE syndrome, was recently shown to be a binding partner of SOX2[65]. SOX2 is linked to AEG syndrome, which is clinically related to CHARGE syndrome. In fact, in some the diagnosis of AEG or CHARGE is hard to distinguish from each other[57,58]. Thus, some of the congenital pulmonary abnormalities may be part of very complex syndromes, and it may be that the interactions between SOX2 and CHD7, or other proteins, may result in combinations of different clinical parameters. Therefore, mutations that change the interactions between SOX2 and other proteins, like CHD7, may result in the various clinical manifestations observed in syndromes.

REFERENCES

1 **Wells JM**, Melton DA. Vertebrate endoderm development. *Annu Rev Cell Dev Biol* 1999; **15**: 393-410 [PMID: 10611967 DOI: 10.1146/annurev.cellbio.15.1.393]

2 **Chamberlain CE**, Jeong J, Guo C, Allen BL, McMahon AP. Notochord-derived Shh concentrates in close association with the apically positioned basal body in neural target cells and forms a dynamic gradient during neural patterning. *Development* 2008; **135**: 1097-1106 [PMID: 18272593 DOI: 10.1242/dev.013086]

3 **Serls AE**, Doherty S, Parvatiyar P, Wells JM, Deutsch GH. Different thresholds of fibroblast growth factors pattern the ventral foregut into liver and lung. *Development* 2005; **132**: 35-47 [PMID: 15576401 DOI: 10.1242/dev.01570]

4 **Desai TJ**, Chen F, Lü J, Qian J, Niederreither K, Dollé P, Chambon P, Cardoso WV. Distinct roles for retinoic acid receptors alpha and beta in early lung morphogenesis. *Dev Biol* 2006; **291**: 12-24 [PMID: 16427040 DOI: 10.1016/j.ydbio.2005.10.045]

5 **Chen F**, Cao Y, Qian J, Shao F, Niederreither K, Cardoso WV. A retinoic acid-dependent network in the foregut controls formation of the mouse lung primordium. *J Clin Invest* 2010; **120**: 2040-2048 [PMID: 20484817]

6 **Morrisey EE**, Hogan BL. Preparing for the first breath: genetic and cellular mechanisms in lung development. *Dev Cell* 2010; **18**: 8-23 [PMID: 20152174 DOI: 10.1016/j.devcel.2009.12.010]

7 **Herriges M**, Morrisey EE. Lung development: orchestrating the generation and regeneration of a complex organ. *Development* 2014; **141**: 502-513 [PMID: 24449833 DOI: 10.1242/dev.098186]

8 **Warburton D**, El-Hashash A, Carraro G, Tiozzo C, Sala F, Rogers O, De Langhe S, Kemp PJ, Riccardi D, Torday J, Bellusci S, Shi W, Lubkin SR, Jesudason E. Lung organogenesis. *Curr Top Dev Biol* 2010; **90**: 73-158 [PMID: 20691848 DOI: 10.1016/S0070-2153(10)90003-3]

9 **Wells JM**, Melton DA. Early mouse endoderm is patterned by soluble factors from adjacent germ layers. *Development* 2000; **127**: 1563-1572 [PMID: 10725233]

10 **Rackley CR**, Stripp BR. Building and maintaining the epithelium of the lung. *J Clin Invest* 2012; **122**: 2724-2730 [PMID: 22850882 DOI: 10.1172/JCI60519]

11 **Burri PH**. Structural aspects of postnatal lung development - alveolar formation and growth. *Biol Neonate* 2006; **89**: 313-322 [PMID: 16770071 DOI: 10.1159/000092868]

12 **Jacobs IJ**, Ku WY, Que J. Genetic and cellular mechanisms regulating anterior foregut and esophageal development. *Dev Biol* 2012; **369**: 54-64 [PMID: 22750256 DOI: 10.1016/j.ydbio.2012.06.016]

13 **Roberts DJ**, Johnson RL, Burke AC, Nelson CE, Morgan BA, Tabin C. Sonic hedgehog is an endodermal signal inducing Bmp-4 and Hox genes during induction and regionalization of the chick hindgut. *Development* 1995; **121**: 3163-3174 [PMID: 7588051]

14 **de Santa Barbara P**, Roberts DJ. Tail gut endoderm and gut/genitourinary/tail development: a new tissue-specific role for Hoxa13. *Development* 2002; **129**: 551-561 [PMID: 11830557]

15 **Litingtung Y**, Lei L, Westphal H, Chiang C. Sonic hedgehog is essential to foregut development. *Nat Genet* 1998; **20**: 58-61 [PMID: 9731532 DOI: 10.1038/1717]

16 **McMahon AP**, Ingham PW, Tabin CJ. Developmental roles and clinical significance of hedgehog signaling. *Curr Top Dev Biol* 2003; **53**: 1-114 [PMID: 12509125 DOI: 10.1016/S0070-2153(03)53002-2]

17 **Moniot B**, Biau S, Faure S, Nielsen CM, Berta P, Roberts DJ, de Santa Barbara P. SOX9 specifies the pyloric sphincter epithelium through mesenchymal-epithelial signals. *Development* 2004; **131**: 3795-3804 [PMID: 15240557 DOI: 10.1242/dev.01259]

18 **de Santa Barbara P**, van den Brink GR, Roberts DJ. Development and differentiation of the intestinal epithelium. *Cell Mol Life Sci* 2003; **60**: 1322-1332 [PMID: 12943221 DOI: 10.1007/s00018-003-2289-3]

19 **Gubbay J**, Collignon J, Koopman P, Capel B, Economou A, Münsterberg A, Vivian N, Goodfellow P, Lovell-Badge R. A gene mapping to the sex-determining region of the mouse Y chromosome is a member of a novel family of embryonically expressed genes. *Nature* 1990; **346**: 245-250 [PMID: 2374589]

20 **Sinclair AH**, Berta P, Palmer MS, Hawkins JR, Griffiths BL, Smith MJ, Foster JW, Frischauf AM, Lovell-Badge R, Goodfellow PN. A gene from the human sex-determining region encodes a protein with homology to a conserved DNA-binding motif. *Nature* 1990; **346**: 240-244 [PMID: 1695712]

21 **Pevny L**, Placzek M. SOX genes and neural progenitor identity. *Curr Opin Neurobiol* 2005; **15**: 7-13 [PMID: 15721738]

22 **Wegner M**. From head to toes: the multiple facets of Sox proteins. *Nucleic Acids Res* 1999; **27**: 1409-1420 [PMID: 10037800]

23 **Harley VR**, Lovell-Badge R, Goodfellow PN. Definition of a consensus DNA binding site for SRY. *Nucleic Acids Res* 1994;

22: 1500-1501 [PMID: 8190643]
24 **Lefebvre V**, Dumitriu B, Penzo-Méndez A, Han Y, Pallavi B. Control of cell fate and differentiation by Sry-related high-mobility-group box (Sox) transcription factors. *Int J Biochem Cell Biol* 2007; **39**: 2195-2214 [PMID: 17625949]
25 **Werner MH**, Burley SK. Architectural transcription factors: proteins that remodel DNA. *Cell* 1997; **88**: 733-736 [PMID: 9118214]
26 **Wolffe AP**. Architectural transcription factors. *Science* 1994; **264**: 1100-1101 [PMID: 8178167]
27 **Pevny LH**, Lovell-Badge R. Sox genes find their feet. *Curr Opin Genet Dev* 1997; **7**: 338-344 [PMID: 9229109]
28 **Wilson M**, Koopman P. Matching SOX: partner proteins and co-factors of the SOX family of transcriptional regulators. *Curr Opin Genet Dev* 2002; **12**: 441-446 [PMID: 12100890]
29 **Sarkar A**, Hochedlinger K. The sox family of transcription factors: versatile regulators of stem and progenitor cell fate. *Cell Stem Cell* 2013; **12**: 15-30 [PMID: 23290134 DOI: 10.1016/j.stem.2012.12.007]
30 **Kamachi Y**, Kondoh H. Sox proteins: regulators of cell fate specification and differentiation. *Development* 2013; **140**: 4129-4144 [PMID: 24086078 DOI: 10.1242/dev.091793]
31 **Goodfellow PN**, Lovell-Badge R. SRY and sex determination in mammals. *Annu Rev Genet* 1993; **27**: 71-92 [PMID: 8122913]
32 **Hacker A**, Capel B, Goodfellow P, Lovell-Badge R. Expression of Sry, the mouse sex determining gene. *Development* 1995; **121**: 1603-1614 [PMID: 7600978]
33 **Koopman P**, Münsterberg A, Capel B, Vivian N, Lovell-Badge R. Expression of a candidate sex-determining gene during mouse testis differentiation. *Nature* 1990; **348**: 450-452 [PMID: 2247150]
34 **Kaplan F**. Molecular determinants of fetal lung organogenesis. *Mol Genet Metab* 2000; **71**: 321-341 [PMID: 11001825 DOI: 10.1006/mgme.2000.3040]
35 **Sock E**, Rettig SD, Enderich J, Bösl MR, Tamm ER, Wegner M. Gene targeting reveals a widespread role for the high-mobility-group transcription factor Sox11 in tissue remodeling. *Mol Cell Biol* 2004; **24**: 6635-6644 [PMID: 15254231 DOI: 10.1128/MCB.24.15.6635-6644.2004]
36 **Lange AW**, Keiser AR, Wells JM, Zorn AM, Whitsett JA. Sox17 promotes cell cycle progression and inhibits TGF-beta/Smad3 signaling to initiate progenitor cell behavior in the respiratory epithelium. *PLoS One* 2009; **4**: e5711 [PMID: 19479035 DOI: 10.1371/journal.pone.0005711]
37 **Tompkins DH**, Besnard V, Lange AW, Keiser AR, Wert SE, Bruno MD, Whitsett JA. Sox2 activates cell proliferation and differentiation in the respiratory epithelium. *Am J Respir Cell Mol Biol* 2011; **45**: 101-110 [PMID: 20855650]
38 **Park KS**, Wells JM, Zorn AM, Wert SE, Whitsett JA. Sox17 influences the differentiation of respiratory epithelial cells. *Dev Biol* 2006; **294**: 192-202 [PMID: 16574095 DOI: 10.1016/j.ydbio.2006.02.038]
39 **Gontan C**, de Munck A, Vermeij M, Grosveld F, Tibboel D, Rottier R. Sox2 is important for two crucial processes in lung development: branching morphogenesis and epithelial cell differentiation. *Dev Biol* 2008; **317**: 296-309 [PMID: 18374910 DOI: 10.1016/j.ydbio.2008.02.035]
40 **Rockich BE**, Hrycaj SM, Shih HP, Nagy MS, Ferguson MA, Kopp JL, Sander M, Wellik DM, Spence JR. Sox9 plays multiple roles in the lung epithelium during branching morphogenesis. *Proc Natl Acad Sci USA* 2013; **110**: E4456-E4464 [PMID: 24191021 DOI: 10.1073/pnas.1311847110]
41 **Turcatel G**, Rubin N, Menke DB, Martin G, Shi W, Warburton D. Lung mesenchymal expression of Sox9 plays a critical role in tracheal development. *BMC Biol* 2013; **11**: 117 [PMID: 24274029 DOI: 10.1186/1741-7007-11-117]
42 **Park J**, Zhang JJ, Moro A, Kushida M, Wegner M, Kim PC. Regulation of Sox9 by Sonic Hedgehog (Shh) is essential for patterning and formation of tracheal cartilage. *Dev Dyn* 2010; **239**: 514-526 [PMID: 20034104 DOI: 10.1002/dvdy.22192]
43 **Elluru RG**, Thompson F, Reece A. Fibroblast growth factor 18 gives growth and directional cues to airway cartilage. *Laryngoscope* 2009; **119**: 1153-1165 [PMID: 19358209 DOI: 10.1002/lary.20157]
44 **Kanai-Azuma M**, Kanai Y, Gad JM, Tajima Y, Taya C, Kurohmaru M, Sanai Y, Yonekawa H, Yazaki K, Tam PP, Hayashi Y. Depletion of definitive gut endoderm in Sox17-null mutant mice. *Development* 2002; **129**: 2367-2379 [PMID: 11973269]
45 **Dong S**, Leung KK, Pelling AL, Lee PY, Tang AS, Heng HH, Tsui LC, Tease C, Fisher G, Steel KP, Cheah KS. Circling, deafness, and yellow coat displayed by yellow submarine (ysb) and light coat and circling (lcc) mice with mutations on chromosome 3. *Genomics* 2002; **79**: 777-784 [PMID: 12036291]
46 **Liu K**, Lin B, Zhao M, Yang X, Chen M, Gao A, Liu F, Que J, Lan X. The multiple roles for Sox2 in stem cell maintenance and tumorigenesis. *Cell Signal* 2013; **25**: 1264-1271 [PMID: 23416461 DOI: 10.1016/j.cellsig.2013.02.013]
47 **Avilion AA**, Nicolis SK, Pevny LH, Perez L, Vivian N, Lovell-Badge R. Multipotent cell lineages in early mouse development depend on SOX2 function. *Genes Dev* 2003; **17**: 126-140 [PMID: 12514105 DOI: 10.1101/gad.224503]
48 **Wood HB**, Episkopou V. Comparative expression of the mouse Sox1, Sox2 and Sox3 genes from pre-gastrulation to early somite stages. *Mech Dev* 1999; **86**: 197-201 [PMID: 10446282]
49 **Papanayotou C**, Mey A, Birot AM, Saka Y, Boast S, Smith JC, Samarut J, Stern CD. A mechanism regulating the onset of Sox2 expression in the embryonic neural plate. *PLoS Biol* 2008; **6**: e2 [PMID: 18184035]
50 **Ishii Y**, Rex M, Scotting PJ, Yasugi S. Region-specific expression of chicken Sox2 in the developing gut and lung epithelium: regulation by epithelial-mesenchymal interactions. *Dev Dyn* 1998; **213**: 464-475 [PMID: 9853967]
51 **Donner AL**, Episkopou V, Maas RL. Sox2 and Pou2f1 interact to control lens and olfactory placode development. *Dev Biol* 2007; **303**: 784-799 [PMID: 17140559]
52 **Schlosser G**, Ahrens K. Molecular anatomy of placode development in Xenopus laevis. *Dev Biol* 2004; **271**: 439-466 [PMID: 15223346]
53 **Uchikawa M**, Ishida Y, Takemoto T, Kamachi Y, Kondoh H. Functional analysis of chicken Sox2 enhancers highlights an array of diverse regulatory elements that are conserved in mammals. *Dev Cell* 2003; **4**: 509-519 [PMID: 12689590]
54 **Taranova OV**, Magness ST, Fagan BM, Wu Y, Surzenko N, Hutton SR, Pevny LH. SOX2 is a dose-dependent regulator of retinal neural progenitor competence. *Genes Dev* 2006; **20**: 1187-1202 [PMID: 16651659]
55 **Pan W**, Jin Y, Chen J, Rottier RJ, Steel KP, Kiernan AE. Ectopic expression of activated notch or SOX2 reveals similar and unique roles in the development of the sensory cell progenitors in the mammalian inner ear. *J Neurosci* 2013; **33**: 16146-16157 [PMID: 24107947 DOI: 10.1523/Jneurosci.3150-12.2013]
56 **Okubo T**, Pevny LH, Hogan BL. Sox2 is required for development of taste bud sensory cells. *Genes Dev* 2006; **20**: 2654-2659 [PMID: 17015430]
57 **Williamson KA**, Hever AM, Rainger J, Rogers RC, Magee A, Fiedler Z, Keng WT, Sharkey FH, McGill N, Hill CJ, Schneider A, Messina M, Turnpenny PD, Fantes JA, van Heyningen V, FitzPatrick DR. Mutations in SOX2 cause anophthalmia-esophageal-genital (AEG) syndrome. *Hum Mol Genet* 2006; **15**: 1413-1422 [PMID: 16543359]
58 **Que J**, Okubo T, Goldenring JR, Nam KT, Kurotani R, Morrisey EE, Taranova O, Pevny LH, Hogan BL. Multiple dose-dependent roles for Sox2 in the patterning and differentiation of anterior foregut endoderm. *Development* 2007; **134**: 2521-2531 [PMID: 17522155 DOI: 10.1242/dev.003855]
59 **Fantes J**, Ragge NK, Lynch SA, McGill NI, Collin JR, Howard-Peebles PN, Hayward C, Vivian AJ, Williamson K, van Heyningen V, FitzPatrick DR. Mutations in SOX2 cause an-

ophthalmia. *Nat Genet* 2003; **33**: 461-463 [PMID: 12612584]
60 **Hagstrom SA**, Pauer GJ, Reid J, Simpson E, Crowe S, Maumenee IH, Traboulsi EI. SOX2 mutation causes anophthalmia, hearing loss, and brain anomalies. *Am J Med Genet A* 2005; **138A**: 95-98 [PMID: 16145681]
61 **Harris-Johnson KS**, Domyan ET, Vezina CM, Sun X. beta-Catenin promotes respiratory progenitor identity in mouse foregut. *Proc Natl Acad Sci USA* 2009; **106**: 16287-16292 [PMID: 19805295 DOI: 10.1073/pnas.0902274106]
62 **Ochieng JK**, Schilders K, Kool H, Boerema-De Munck A, Buscop-Van Kempen M, Gontan C, Smits R, Grosveld FG, Wijnen RM, Tibboel D, Rottier RJ. Sox2 regulates the emergence of lung basal cells by directly activating the transcription of Trp63. *Am J Respir Cell Mol Biol* 2014; **51**: 311-322 [PMID: 24669837 DOI: 10.1165/rcmb.2013-0419OC]
63 **Minoo P**, Su G, Drum H, Bringas P, Kimura S. Defects in tracheoesophageal and lung morphogenesis in Nkx2.1(-/-) mouse embryos. *Dev Biol* 1999; **209**: 60-71 [PMID: 10208743 DOI: 10.1006/dbio.1999.9234]
64 **Boyer LA**, Lee TI, Cole MF, Johnstone SE, Levine SS, Zucker JP, Guenther MG, Kumar RM, Murray HL, Jenner RG, Gifford DK, Melton DA, Jaenisch R, Young RA. Core transcriptional regulatory circuitry in human embryonic stem cells. *Cell* 2005; **122**: 947-956 [PMID: 16153702 DOI: 10.1016/j.cell.2005.08.020]
65 **Engelen E**, Akinci U, Bryne JC, Hou J, Gontan C, Moen M, Szumska D, Kockx C, van Ijcken W, Dekkers DH, Demmers J, Rijkers EJ, Bhattacharya S, Philipsen S, Pevny LH, Grosveld FG, Rottier RJ, Lenhard B, Poot RA. Sox2 cooperates with Chd7 to regulate genes that are mutated in human syndromes. *Nat Genet* 2011; **43**: 607-611 [PMID: 21532573 DOI: 10.1038/Ng.825]
66 **Gontan C**, Güttler T, Engelen E, Demmers J, Fornerod M, Grosveld FG, Tibboel D, Görlich D, Poot RA, Rottier RJ. Exportin 4 mediates a novel nuclear import pathway for Sox family transcription factors. *J Cell Biol* 2009; **185**: 27-34 [PMID: 19349578 DOI: 10.1016/j.ydbio.2008.05.306]
67 **Bellusci S**, Grindley J, Emoto H, Itoh N, Hogan BL. Fibroblast growth factor 10 (FGF10) and branching morphogenesis in the embryonic mouse lung. *Development* 1997; **124**: 4867-4878 [PMID: 9428423]
68 **Park WY**, Miranda B, Lebeche D, Hashimoto G, Cardoso WV. FGF-10 is a chemotactic factor for distal epithelial buds during lung development. *Dev Biol* 1998; **201**: 125-134 [PMID: 9740653]
69 **Sekine K**, Ohuchi H, Fujiwara M, Yamasaki M, Yoshizawa T, Sato T, Yagishita N, Matsui D, Koga Y, Itoh N, Kato S. Fgf10 is essential for limb and lung formation. *Nat Genet* 1999; **21**: 138-141 [PMID: 9916808 DOI: 10.1038/5096]
70 **Kato S**, Sekine K. [FGF10, a key factor for limb and lung formation]. *Seikagaku* 2000; **72**: 288-291 [PMID: 10853366]
71 **Colvin JS**, White AC, Pratt SJ, Ornitz DM. Lung hypoplasia and neonatal death in Fgf9-null mice identify this gene as an essential regulator of lung mesenchyme. *Development* 2001; **128**: 2095-2106 [PMID: 11493531]
72 **Abler LL**, Mansour SL, Sun X. Conditional gene inactivation reveals roles for Fgf10 and Fgfr2 in establishing a normal pattern of epithelial branching in the mouse lung. *Dev Dyn* 2009; **238**: 1999-2013 [PMID: 19618463 DOI: 10.1002/dvdy.22032]
73 **Krejci P**, Aklian A, Kaucka M, Sevcikova E, Prochazkova J, Masek JK, Mikolka P, Pospisilova T, Spoustova T, Weis M, Paznekas WA, Wolf JH, Gutkind JS, Wilcox WR, Kozubik A, Jabs EW, Bryja V, Salazar L, Vesela I, Balek L. Receptor tyrosine kinases activate canonical WNT/β-catenin signaling via MAP kinase/LRP6 pathway and direct β-catenin phosphorylation. *PLoS One* 2012; **7**: e35826 [PMID: 22558232 DOI: 10.1371/journal.pone.0035826]
74 **Shu W**, Guttentag S, Wang Z, Andl T, Ballard P, Lu MM, Piccolo S, Birchmeier W, Whitsett JA, Millar SE, Morrisey EE. Wnt/beta-catenin signaling acts upstream of N-myc, BMP4, and FGF signaling to regulate proximal-distal patterning in the lung. *Dev Biol* 2005; **283**: 226-239 [PMID: 15907834 DOI: 10.1016/j.ydbio.2005.04.014]
75 **Cornett B**, Snowball J, Varisco BM, Lang R, Whitsett J, Sinner D. Wntless is required for peripheral lung differentiation and pulmonary vascular development. *Dev Biol* 2013; **379**: 38-52 [PMID: 23523683 DOI: 10.1016/j.ydbio.2013.03.010]
76 **Goss AM**, Tian Y, Tsukiyama T, Cohen ED, Zhou D, Lu MM, Yamaguchi TP, Morrisey EE. Wnt2/2b and beta-catenin signaling are necessary and sufficient to specify lung progenitors in the foregut. *Dev Cell* 2009; **17**: 290-298 [PMID: 19686689 DOI: 10.1016/j.devcel.2009.06.005]
77 **Shu W**, Jiang YQ, Lu MM, Morrisey EE. Wnt7b regulates mesenchymal proliferation and vascular development in the lung. *Development* 2002; **129**: 4831-4842 [PMID: 12361974]
78 **Miller MF**, Cohen ED, Baggs JE, Lu MM, Hogenesch JB, Morrisey EE. Wnt ligands signal in a cooperative manner to promote foregut organogenesis. *Proc Natl Acad Sci USA* 2012; **109**: 15348-15353 [PMID: 22949635 DOI: 10.1073/pnas.1201583109]
79 **Hashimoto S**, Chen H, Que J, Brockway BL, Drake JA, Snyder JC, Randell SH, Stripp BR. β-Catenin-SOX2 signaling regulates the fate of developing airway epithelium. *J Cell Sci* 2012; **125**: 932-942 [PMID: 22421361 DOI: 10.1242/jcs.092734]
80 **Mucenski ML**, Nation JM, Thitoff AR, Besnard V, Xu Y, Wert SE, Harada N, Taketo MM, Stahlman MT, Whitsett JA. Beta-catenin regulates differentiation of respiratory epithelial cells in vivo. *Am J Physiol Lung Cell Mol Physiol* 2005; **289**: L971-L979 [PMID: 16040629 DOI: 10.1152/ajplung.00172.2005]
81 **Li C**, Hu L, Xiao J, Chen H, Li JT, Bellusci S, Delanghe S, Minoo P. Wnt5a regulates Shh and Fgf10 signaling during lung development. *Dev Biol* 2005; **287**: 86-97 [PMID: 16169547]
82 **Sountoulidis A**, Stavropoulos A, Giaglis S, Apostolou E, Monteiro R, Chuva de Sousa Lopes SM, Chen H, Stripp BR, Mummery C, Andreakos E, Sideras P. Activation of the canonical bone morphogenetic protein (BMP) pathway during lung morphogenesis and adult lung tissue repair. *PLoS One* 2012; **7**: e41460 [PMID: 22916109 DOI: 10.1371/journal.pone.0041460]
83 **Que J**, Choi M, Ziel JW, Klingensmith J, Hogan BL. Morphogenesis of the trachea and esophagus: current players and new roles for noggin and Bmps. *Differentiation* 2006; **74**: 422-437 [PMID: 16916379 DOI: 10.1111/j.1432-0436.2006.00096.x]
84 **Li Y**, Litingtung Y, Ten Dijke P, Chiang C. Aberrant Bmp signaling and notochord delamination in the pathogenesis of esophageal atresia. *Dev Dyn* 2007; **236**: 746-754 [PMID: 17260385 DOI: 10.1002/dvdy.21075]
85 **Fausett SR**, Brunet LJ, Klingensmith J. BMP antagonism by Noggin is required in presumptive notochord cells for mammalian foregut morphogenesis. *Dev Biol* 2014; **391**: 111-124 [PMID: 24631216 DOI: 10.1016/j.ydbio.2014.02.008]
86 **Li Y**, Gordon J, Manley NR, Litingtung Y, Chiang C. Bmp4 is required for tracheal formation: a novel mouse model for tracheal agenesis. *Dev Biol* 2008; **322**: 145-155 [PMID: 18692041 DOI: 10.1016/j.ydbio.2008.07.021]
87 **Domyan ET**, Ferretti E, Throckmorton K, Mishina Y, Nicolis SK, Sun X. Signaling through BMP receptors promotes respiratory identity in the foregut via repression of Sox2. *Development* 2011; **138**: 971-981 [PMID: 21303850 DOI: 10.1242/dev.053694]
88 **Correia-Pinto J**, Gonzaga S, Huang Y, Rottier R. Congenital lung lesions--underlying molecular mechanisms. *Semin Pediatr Surg* 2010; **19**: 171-179 [PMID: 20610189 DOI: 10.1053/j.sempedsurg.2010.03.003]
89 **Ng C**, Stanwell J, Burge DM, Stanton MP. Conservative management of antenatally diagnosed cystic lung malformations. *Arch Dis Child* 2014; **99**: 432-437 [PMID: 24406806 DOI: 10.1136/archdischild-2013-304048]
90 **Langston C**. New concepts in the pathology of congenital

lung malformations. *Semin Pediatr Surg* 2003; **12**: 17-37 [PMID: 12520470 DOI: 10.1053/spsu.2003.00001]

91 **Cass DL**, Quinn TM, Yang EY, Liechty KW, Crombleholme TM, Flake AW, Adzick NS. Increased cell proliferation and decreased apoptosis characterize congenital cystic adenomatoid malformation of the lung. *J Pediatr Surg* 1998; **33**: 1043-1046; discussion 1047 [PMID: 9694091]

92 **Fromont-Hankard G**, Philippe-Chomette P, Delezoide AL, Nessmann C, Aigrain Y, Peuchmaur M. Glial cell-derived neurotrophic factor expression in normal human lung and congenital cystic adenomatoid malformation. *Arch Pathol Lab Med* 2002; **126**: 432-436 [PMID: 11900567 DOI: 10.1043/0003-9985(2002)126<0432:GCDNFE>2.0.CO;2]

93 **Gonzaga S**, Henriques-Coelho T, Davey M, Zoltick PW, Leite-Moreira AF, Correia-Pinto J, Flake AW. Cystic adenomatoid malformations are induced by localized FGF10 overexpression in fetal rat lung. *Am J Respir Cell Mol Biol* 2008; **39**: 346-355 [PMID: 18421016 DOI: 10.1165/rcmb.2007-0290OC]

94 **Volpe MV**, Pham L, Lessin M, Ralston SJ, Bhan I, Cutz E, Nielsen HC. Expression of Hoxb-5 during human lung development and in congenital lung malformations. *Birth Defects Res A Clin Mol Teratol* 2003; **67**: 550-556 [PMID: 14632303 DOI: 10.1002/bdra.10086]

95 **Jancelewicz T**, Nobuhara K, Hawgood S. Laser microdissection allows detection of abnormal gene expression in cystic adenomatoid malformation of the lung. *J Pediatr Surg* 2008; **43**: 1044-1051 [PMID: 18558180 DOI: 10.1016/j.jpedsurg.2008.02.027]

96 **Wagner AJ**, Stumbaugh A, Tigue Z, Edmondson J, Paquet AC, Farmer DL, Hawgood S. Genetic analysis of congenital cystic adenomatoid malformation reveals a novel pulmonary gene: fatty acid binding protein-7 (brain type). *Pediatr Res* 2008; **64**: 11-16 [PMID: 18391847 DOI: 10.1203/PDR.0b013e318174eff8]

97 **Volpe MV**, Archavachotikul K, Bhan I, Lessin MS, Nielsen HC. Association of bronchopulmonary sequestration with expression of the homeobox protein Hoxb-5. *J Pediatr Surg* 2000; **35**: 1817-1819 [PMID: 11101743 DOI: 10.1053/jpsu.2000.19266]

98 **Volpe MV**, Vosatka RJ, Nielsen HC. Hoxb-5 control of early airway formation during branching morphogenesis in the developing mouse lung. *Biochim Biophys Acta* 2000; **1475**: 337-345 [PMID: 10913834]

99 **Berg C**, Geipel A, Germer U, Pertersen-Hansen A, Koch-Dörfler M, Gembruch U. Prenatal detection of Fraser syndrome without cryptophthalmos: case report and review of the literature. *Ultrasound Obstet Gynecol* 2001; **18**: 76-80 [PMID: 11489232 DOI: 10.1046/j.1469-0705.2001.00374.x]

100 **Epelman M**, Kreiger PA, Servaes S, Victoria T, Hellinger JC. Current imaging of prenatally diagnosed congenital lung lesions. *Semin Ultrasound CT MR* 2010; **31**: 141-157 [PMID: 20304322 DOI: 10.1053/j.sult.2010.01.002]

101 **Mei-Zahav M**, Konen O, Manson D, Langer JC. Is congenital lobar emphysema a surgical disease? *J Pediatr Surg* 2006; **41**: 1058-1061 [PMID: 16769334 DOI: 10.1016/j.jpedsurg.2006.02.011]

102 **Nayar PM**, Thakral CL, Sajwani MJ. Congenital lobar emphysema and sequestration--treatment by embolization. *Pediatr Surg Int* 2005; **21**: 727-729 [PMID: 15995872 DOI: 10.1007/s00383-005-1462-1]

103 **Kravitz RM**. Congenital malformations of the lung. *Pediatr Clin North Am* 1994; **41**: 453-472 [PMID: 8196988]

104 **Rottier R**, Tibboel D. Fetal lung and diaphragm development in congenital diaphragmatic hernia. *Semin Perinatol* 2005; **29**: 86-93 [PMID: 16050526]

105 **Klaassens M**, de Klein A, Tibboel D. The etiology of congenital diaphragmatic hernia: still largely unknown? *Eur J Med Genet* 2009; **52**: 281-286 [PMID: 19464395 DOI: 10.1016/j.ejmg.2009.05.005]

106 **Beurskens N**, Klaassens M, Rottier R, de Klein A, Tibboel D. Linking animal models to human congenital diaphragmatic hernia. *Birth Defects Res A Clin Mol Teratol* 2007; **79**: 565-572 [PMID: 17469205 DOI: 10.1002/bdra.20370]

107 **Veenma DC**, de Klein A, Tibboel D. Developmental and genetic aspects of congenital diaphragmatic hernia. *Pediatr Pulmonol* 2012; **47**: 534-545 [PMID: 22467525 DOI: 10.1002/ppul.22553]

108 **Leeuwen L**, Fitzgerald DA. Congenital diaphragmatic hernia. *J Paediatr Child Health* 2014; **50**: 667-673 [PMID: 24528549 DOI: 10.1111/jpc.12508]

109 **Brady PD**, Moerman P, De Catte L, Deprest J, Devriendt K, Vermeesch JR. Exome sequencing identifies a recessive PIGN splice site mutation as a cause of syndromic congenital diaphragmatic hernia. *Eur J Med Genet* 2014; **57**: 487-493 [PMID: 24852103 DOI: 10.1016/j.ejmg.2014.05.001]

110 **Brady PD**, Van Houdt J, Callewaert B, Deprest J, Devriendt K, Vermeesch JR. Exome sequencing identifies ZFPM2 as a cause of familial isolated congenital diaphragmatic hernia and possibly cardiovascular malformations. *Eur J Med Genet* 2014; **57**: 247-252 [PMID: 24769157 DOI: 10.1016/j.ejmg.2014.04.006]

111 **Torfs CP**, Curry CJ, Bateson TF. Population-based study of tracheoesophageal fistula and esophageal atresia. *Teratology* 1995; **52**: 220-232 [PMID: 8838292 DOI: 10.1002/tera.1420520408]

112 **Spitz L**. Oesophageal atresia. *Orphanet J Rare Dis* 2007; **2**: 24 [PMID: 17498283 DOI: 10.1186/1750-1172-2-24]

113 **de Jong EM**, Felix JF, de Klein A, Tibboel D. Etiology of esophageal atresia and tracheoesophageal fistula: "mind the gap". *Curr Gastroenterol Rep* 2010; **12**: 215-222 [PMID: 20425471 DOI: 10.1007/s11894-010-0108-1]

114 **Bishop NB**, Stankiewicz P, Steinhorn RH. Alveolar capillary dysplasia. *Am J Respir Crit Care Med* 2011; **184**: 172-179 [PMID: 21471096 DOI: 10.1164/rccm.201010-1697CI]

115 **Kalinichenko VV**, Lim L, Shin B, Costa RH. Differential expression of forkhead box transcription factors following butylated hydroxytoluene lung injury. *Am J Physiol Lung Cell Mol Physiol* 2001; **280**: L695-L704 [PMID: 11238010]

116 **Mahlapuu M**, Enerbäck S, Carlsson P. Haploinsufficiency of the forkhead gene Foxf1, a target for sonic hedgehog signaling, causes lung and foregut malformations. *Development* 2001; **128**: 2397-2406 [PMID: 11493558]

117 **Kiefer JC**. Back to basics: Sox genes. *Dev Dyn* 2007; **236**: 2356-2366 [PMID: 17584862 DOI: 10.1002/dvdy.21218]

118 **Rainger JK**, Bhatia S, Bengani H, Gautier P, Rainger J, Pearson M, Ansari M, Crow J, Mehendale F, Palinkasova B, Dixon MJ, Thompson PJ, Matarin M, Sisodiya SM, Kleinjan DA, Fitzpatrick DR. Disruption of SATB2 or its long-range cis-regulation by SOX9 causes a syndromic form of Pierre Robin sequence. *Hum Mol Genet* 2014; **23**: 2569-2579 [PMID: 24363063 DOI: 10.1093/hmg/ddt647]

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MINIREVIEWS

Preimplantation HLA typing: Practical tool for stem cell transplantation treatment of congenital disorders

Anver Kuliev, Svetlana Rechitsky

Anver Kuliev, Svetlana Rechitsky, Reproductive Genetics Institute, Chicago, IL 60657, United States
Author contributions: Kuliev A and Rechitsky S contributed equally to this work, generated the design and wrote the manuscript.
Correspondence to: Anver Kuliev, MD, PhD, Reproductive Genetics Institute, 2825 N Halsted St, Chicago, IL 60657, United States. anverkuliev@hotmail.com
Telephone: +1-847-4001515 Fax: +1-847-4001516
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Abstract

It is well known that to achieve an acceptable engraftment and survival in stem cell therapy, an human leukocyte antigens (HLA) identical stem cell transplant is strongly required. However, the availability of the HLA matched donors even among family members is extremely limited, so preimplantation HLA typing provides an attractive practical tool of stem cell therapy for children requiring HLA matched stem cell transplantation. The present experience of preimplantation genetic diagnosis (PGD) for HLA typing of over one thousand cases shows that PGD provides the at-risk couples with the option to establish an unaffected pregnancy, which may benefit the affected member of the family with hemoglobinopathies, immunodeficiencies and other congenital or acquired bone marrow failures. Despite ethical issues involved in preimplantation HLA typing, the data presented below show an extremely high attractiveness of this option for the couples with affected children requiring HLA compatible stem cell transplantation.

Key words: Preimplantation HLA typing; Preimplantation genetic diagnosis; Stem cell transplantation; Hemoglobinopathies; Immunodeficiencies; Aneuploidy testing

Core tip: Human leukocyte antigens (HLA) identical stem cell transplant is the key in achieving an acceptable engraftment and survival in stem cell therapy. However, the availability of the HLA matched donors even among family members is extremely limited, so preimplantation HLA typing provides an attractive practical tool of stem cell therapy for children requiring HLA matched stem cell transplantation.

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INTRODUCTION

Preimplantation human leukocyte antigens (HLA) matching could not be indication for prenatal diagnosis because it is not acceptable to terminate a normal pregnancy only because the foetus is HLA unmatched. However, preimplantation genetic diagnosis (PGD) for this purpose is totally different, as maximum two embryos are transferred so these embryos may be pre-selected to be unaffected one and match to affected siblings to perform HLA matched stem cell transplantation. This was first introduced in combination with mutation analysis for Fanconi anemia (FA), with a total success[1,2]. In fact FA was also the first disorder for which cord blood stem cell transplantation has been first performed[3]. FA is genetically heterogeneous group of disorders, involving different complementation groups (FANCA, FANCB, FANCC, FANCD and FANCE)[4-6], for which stem cell transplantation is the only treatment as the objective is to restor hematopoiesis which can be done only with HLA identical stem cells, to prevent severe graft *vs* host disease[7,8].

As will be described in this paper, preimplantation

Table 1 Experience in preimplantation genetic diagnosis with HLA typing

Disease	Patients	Cycles	No. of embryo transfers	No. embryos transferred	Pregnancy	Birth
Thalassemia/sickle cell disease	51	149	82	130	20	15
FANCA, FANCC, FANCD2, FANCF, FANCI, FANCJ	17	53	34	52	7	4
WAS	2	2	2	4	1	1
X-ALD	2	5	1	1	0	0
Hyper IgM	5	8	6	9	3	2
HED-ID	2	9	6	8	2	3
DBA	3	5	3	6	2	2
Krabbe	1	1	1	2	1	2
MD	1	2	1	2	1	2
Chronic granulomatous disease	1	3	3	5	1	1
Total	85	238	139	219	38	32

WAS: Wiscott-aldrich syndrome; X-ALD: X-linked adrenoleukodystrophy; HED-ID: Hypohidrotic ectodermal displasia with immune deficiency; DBA: Diamond-Blackfan anemia; MD: Muscular dystrophy.

Table 2 Chances for detection of disease free and HLA match embryo in preimplantation HLA typing

HLA MATCH only-1/4 (25%)
Autosomal recessive or X-linked free + HLA MATCH-3/4 × 1/4 = 3/16 (18.75%)
Autosomal dominat free + HLA MATCH-1/2 × 1/4 = 1/8 (12.5%)
Autosomal recessive or X-linked free + HLA MATCH + ANEUPLOIDY-free-3/4 × 1/4 × 1/2 = 3/32 (9.4%)
Autosomal dominant free + HLA MATCH + ANEUPLOIDY-free-1/2 × 1/4 × 1/2 = 1/16 (6.25%)

HLA: Human leukocyte antigens.

HLA testing is currently applied not only with PGD for single gene disorders, but also as a sole indication.

PREIMPLANTATION HLA TYPING WITH AND WITHOUT PGD FOR SINGLE GENE DISORDERS

Our experience on PGD with HLA typing is presented in Table 1, showing that among conditions requiring HLA compatible stem cell transplantation, hemoglobinopathies are the major indication, representing the commonest autosomal recessive diseases in Mediterranean region, Middle East and South East Asia.

Hemoglobinopathies

Hemoglobinopathies, including thalassemia and sickle cell disease, are autosomal recessive disorders with abnormal production of beta-globin chains that leads to a severe anaemia, requiring a life-long blood transfusion Prevention of these disorders has been done using fetal diagnosis with reduction of new cases of thalassemia to up to 70% in many populations, including such large countries in the Eastern Mediterranean region, as Greece, Turkey and Iran[9-11]. There has been progress also in tin treatment by bone marrow transplantation[12], but this is limited due to unavailability of HLA matched stem cells, that can be overcome by PGD. We introduced PGD for thalassemia 18 years ago[13-15], and HLA typing is actually a natural extention allowing couples to produce an unaffected child as a potential HLA matched donor for thalassemic sibling.

In our experience, of a total of 293 PGD cycles for 161 couples at risk for producing offspring with hemoglobinopathies, 149 cycles were performed for HLA typing. Polar body (PB) or embryo biopsy was used to identify hemoglobinopathy mutations, and embryo biopsy was also used for HLA testing, in order to identify the embryos containing the maternal and paternal chromosomes 6 identical to the affected siblings, as described in detail elsewhere[16-18].

HLA typing was based on the methods described elsewhere[19-22]. The chances to identify unaffected embryos fully matched to thalassemic siblings is 18.75%, as for other autosomal recessive conditions, based on 25% chance of HLA match and 75% chance of having unaffected embryo (Table 2).

Of more than two dozens of different beta-globin gene mutations tested, the most frequent ones were *IVSI-110* mutation -100 cases (33%), followed by IVSI-6-39 cases, IVSII-745-23 cases, Codon 8-20 cases, IVSI-1-18 cases, and codon 39 and IVSI-5-16 cases each. Among other mutations were *IVSII-2*, *Codon 5*, *Codon 6*, *Codon 41/2*, *E121K*, -29 (A-G)-87, *R30T*, *Cap 1*, deletion 69 kb and deletion 13.4 kb. Mutation testing resulted in detection and transfer of 476 unaffected embryos (approximately, 2 embryos per transfer) in 240 (81.9%) of 293 clinical cycles, yielding 67 (27.9%) unaffected pregnancies and birth of 70 thalassemia-free children[18]. PGD for thalassemias currently represents approximately 15% of our PGD series of 2028 cases, which is the world's largest series for monogenic conditions[23].

A total of 149 of these PGD cycles were performed for HLA typing, which allowed detecting and transferring unaffected HLA matched embryos in 82 of them (Table 1). Of 824 embryos with conclusive results for testing of beta-globin gene mutations and HLA type, 602 (73.0%) were predicted to be unaffected carriers or normal, of which only 130 (15.8%) appeared to be HLA identical to

the affected siblings, which, as mentioned, is not significantly different from the expectation (Table 2)[18]. These embryos were replaced, yielding 20 healthy matched clinical pregnancies. Umbilical cord blood was collected at birth of these children, or bone marrow obtained at 1 year of age, and transplanted or pending, resulting in a successful hematopoietic reconstitution in all of them. Clearly the progress in radical treatment of hemoglobinopathies will depend on the availability of HLA identical donors[24].

PGD for HLA typing has currently been applied as an efficient tool for couples at risk in many PGD centres to ensure having thalassemia-free children who are HLA identical to the affected siblings, to serve a potential donor for stem cells for transplantation treatment. This currently is a practical tool for a use in communities where hemoglobinopathies are endemic and will improve the access to HLA matched bone marrow transplantation of these prevalent conditions.

The other large series of PGD for HLA typing for thalassemia was reported from Turkey, where 236 PGD cycles were performed resulting in birth of 70 thalassemia-free children. Of 48 affected children transplanted (in addition to thalassemia, morbid children with 9 other different conditions was transplanted), successful outcome was observed in 44 of them with a total hematopoietic reconstitution, while the graft failure occurred only in 4 of them[25-27].

Immunodeficiencies

Preimplantation HLA typing appeared to be of great utility for severe congenital immunodeficiencies (SCID), which is the key in in finding matched stem cell transplant to save live of SCID patients. Our accumulated series on PGD for SCID is presented in Table 1[28]. A total of 19 PGD cycles for 9 couples for producing affected progeny with the above conditions (this does not include PGD cycles for FA, which will be described below) were performed, including 8 cycles for Hyper IgM (HIGM), 2 for wiscott-aldrich syndrome (WAS), and 9 for hypohidrotic ectodermal displasia with immune deficiency (HED-ID). The Table 1 does not include three cases of PGD for AT and one for omen syndrome (OMS), which were performed without HLA typing, as the affected children did not survive by the time of performing PGD. PGD for OMS was the world's first case, which resulted in transfer of two unaffected embryos, yielding the birth of healthy twins. As mentioned, there was no need for HLA typing in this particular case, but the couples with previous OMS children will definitely be potential candidates for performing PGD with HLA typing to provide also an identical HLA donor progeny for stem cell transplantation. This is also highly relevant to ataxia-telangiectasia (AT), which is a progressive, neurodegenerative childhood disease that affects the brain and other body systems. A weakened immune system makes the patients susceptible to recurrent respiratory infections. Although the currently used symptomatic and supportive treatment, including high-dose vitamin regimens, physical and occupational therapy and gamma-globulin injections to supplement a weakened immune system may be helpful, the prognosis is very poor, patients still dying in their teens.

A single case PGD for AT has been reported previously for a Saudi patient with 3 affected children[29]. The disease was caused by a large deletion of more than two thirds of the *AT* gene, which was detected by amplification of one of the deleted exons (exon 19). Of three embryos available for biopsy and testing, one was a deletion free and transferred, resulting in an unaffected pregnancy.

Of 17 couples at risk for producing a progeny with FA, in addition to two carriers of IVS 4+4 A-T mutation in *FANCC* gene, three were carriers of *FANCD2*, *FANCF*, *FANCI*, *FAMCCJ*, and *FANCA* gene mutations. Overall, 52 unaffected HLA matched embryos were transferred in 34 of 53 initiated cycles, resulting in seven unaffected pregnancies and 4 FA free and HLA matched children , as potential donors for their siblings.

Five cycles were performed for X-linked Adrenoleukodystrophy, which affects the nervous system and the adrenal cortex. Of special interest is preimplantation HLA typing for HIGM, which is a rare immunodeficiency characterized by normal or elevated serum IgM levels, with absence of IgG, IgA and IgE, which results in an increased susceptibility to infections.

Of 5 couples with HIGM for whom PGD was performed, one was with C218X mutation in exon 5 of CD40 ligant gene, 3 with maternal mutations C218X exon 4 c.437_38 ins A, and one with exon 4 c.397 ins T. The maternal mutations were analyzed by PB1 and PB2, followed by HLA and aneuploidy testing in biopsied blastomeres. Of 8 PGD cycles for HLA performed, 9 unaffected HLA matched embryos were transferred in 6 cycles, resulting in 3 clinical pregnancies and birth of 2 healthy babies, as potential donors of HLA compatible stem cells for their siblings.

The first attempt of cord blood transplantation from one of the babies did not result in acceptable engraftment, so the second transplantation was performed one year later, using bone marrow mixed with the remaining portion of the cord blood sample, which provided better results in achieving successful engraftment and reconstitution of the sibling's bone marrow, and resulting in a total cure of the patient.

A total of 11 cycles were performed for WAS and X-linked HED-ID, in which 12 embryos were detected to be unaffected and HLA matched (8 for HED-ID and 4 for WAS), and transferred in 8 cycles, resulting in birth of 4 unaffected babies (3 free of HED-ID and 1 free of WAS), confirmed to be HLA matched to affected sibling.

Preimplantation HLA typing as a sole indicator

As presented in Table 3, in addition to 238 PGD for HLA cycles, 98 cycles were performed for preimplantation HLA matching without testing for causative gene. These couples were wishing to have another child anyway, but requested that if these children could become a

Table 3 Preimplantation HLA typing with and without preimplantation genetic diagnosis

Preimplantation testing	Patients	Cycles	No. of embryo transfers	No. embryos transferred	Pregnancy/birth
HLA TESTING ONLY	46	98	65	99	24/19
HLA + MUTATION	85	238	139	219	38/ 32
Total	131	336	204	318	62/51

HLA: Human leukocyte antigens.

source of stem cell transplant to save live of siblings with acquired bane marrow failures, such as sporadic Diamond- Blackfan anemia[30].

There was no difference in performing preimplantation HLA testing without PGD, except limiting the analysis of the day 3 or day 5 embryos to only HLA typing, with the sibling requiring stem cell transplantation, using a multiplex hemi-nested PCR system.

In a total of 98 clinical cycles from 46 couples performed with a primary indication of HLA typing, 99 HLA matched embryos were pre-selected for transfer. Proportion of embryos predicted to be HLA matched to the affected siblings was 21.5%, not significantly different from the expected 25% (Table 2). The transfer of 99 HLA matched embryos in 65 clinical cycles, resulted in 24 pregnancies and 19 HLA identicaldeliveries, with already available results of complete cure[30].

LIMITATIONS AND FUTURE PROSPECT OF PGD FOR HLA TYPING

A relatively high frequency of recombination in the HLA region is one of the major limitations of PGD for HLA typing, which may affect not only the accuracy of preimplantation HLA typing, but also the outcome of stem cell transplantation. In our experience, of 1713 embryos tested for HLA, 1634 (95.5%) were non-recombinant, 52 (3%) with maternal, and 27 (1.5%) with paternal recombination.. The major problem in performing PGD for HLA may be faced when the preparatory testing identified the sibling being with maternal recombination, so it could be unrealistic to identify the exact match, so the couples should be informed that only relatively close match may be possible, which may be discussed with paediatric haematologist in the pre-selection process of the embryos for transfer.

The other important limitation is that the majority of cases are in couples of advanced maternal age, so aneuploidy testing is usually an integral part of the procedure. Although the chances of pre-selecting unaffected HLA matched embryos that could be also euploid is quite low, our preliminary results of the reproductive outcome comparison between the groups of combined PGD/HLA with and without aneuploidy testing showed a significant difference. Despite transferring a lower number of embryos, the pregnancy rate was higher in the aneuploidy testing group, suggesting the potential utility of aneuploidy testing in preimplantation HLA typing, allowing the avoidance of transfer of those HLA identical embryos that are chromosomally abnormal, which are destined to be lost anyway either before or after implantation.

Therefore, patients should be properly counseled to be aware of the limits of the procedure and even lower proportion of available embryos for transfer than may have been predicted, depending also on the maternal age.

REFERENCES

1 **Verlinsky Y**, Rechitsky S, Schoolcraft W, Strom C, Kuliev A. Designer babies - are they a reality yet? Case report: simultaneous preimplantation genetic diagnosis for Fanconi anaemia and HLA typing for cord blood transplantation. *Reprod Biomed Online* 2000; **1**: 31 [PMID: 12804194 DOI: 10.1016/S1472-6483(10)61896-2]

2 **Verlinsky Y**, Rechitsky S, Schoolcraft W, Strom C, Kuliev A. Preimplantation diagnosis for Fanconi anemia combined with HLA matching. *JAMA* 2001; **285**: 3130-3133 [PMID: 11427142 DOI: 10.1001/jama.285.24.3130]

3 **Gluckman E**, Broxmeyer HA, Auerbach AD, Friedman HS, Douglas GW, Devergie A, Esperou H, Thierry D, Socie G, Lehn P. Hematopoietic reconstitution in a patient with Fanconi's anemia by means of umbilical-cord blood from an HLA-identical sibling. *N Engl J Med* 1989; **321**: 1174-1178 [PMID: 2571931 DOI: 10.1056/NEJM198910263211707]

4 **Strathdee CA**, Duncan AM, Buchwald M. Evidence for at least four Fanconi anaemia genes including FACC on chromosome 9. *Nat Genet* 1992; **1**: 196-198 [PMID: 1303234 DOI: 10.1038/ng0692-196]

5 **Strathdee CA**, Gavish H, Shannon WR, Buchwald M. Cloning of cDNAs for Fanconi's anaemia by functional complementation. *Nature* 1992; **356**: 763-767 [PMID: 1574115 DOI: 10.1038/356763a0]

6 **Whitney MA**, Saito H, Jakobs PM, Gibson RA, Moses RE, Grompe M. A common mutation in the FACC gene causes Fanconi anaemia in Ashkenazi Jews. *Nat Genet* 1993; **4**: 202-205 [PMID: 8348157 DOI: 10.1038/ng0693-202]

7 **Gluckman E**, Devergie A, Schaison G, Bussel A, Berger R, Sohier J, Bernard J. Bone marrow transplantation in Fanconi anaemia. *Br J Haematol* 1980; **45**: 557-564 [PMID: 7000153 DOI: 10.1111/j.1365-2141.1980.tb07178.x]

8 **Wagner J**, Davies SM, Auerbach AD. Hemotopoietic stem cell transplantation in the treatment of Fanconi anemia. In: Forman SJ, Blum KG, Thomas ED, eds. Hematopoietic Cell Transplantation. 2nd ed. Malden, Mass: Blackwell Science Inc, 1999: 1204-1219

9 **Modell B**, Kuliev A. The history of community genetics: the contribution of the haemoglobin disorders. *Community Genet* 1998; **1**: 3-11 [PMID: 15178981 DOI: 10.1159/000016129]

10 **Canatan D**. Hemoglobinopathy prevention programme in Turkey. *Thalassemia Reports* 2011; **1**: 2

11 **Samavat A**. Thalassemia prevention programme: A Model from Iran. *Thalassemia Reports* 2011; **1**: 2

12 **Lucarelli G**, Andreani M, Angelucci E. The cure of thalassemia by bone marrow transplantation. *Blood Rev* 2002; **16**: 81-85 [PMID: 12127951 DOI: 10.1054/blre.2002.0192]

13 **Kuliev A**, Rechitsky S, Verlinsky O, Ivakhnenko V, Evsikov S, Wolf G, Angastiniotis M, Georghiou D, Kukharenko V,

Strom C, Verlinsky Y. Preimplantation diagnosis of thalassemias. *J Assist Reprod Genet* 1998; **15**: 219-225 [PMID: 9604751]

14 **Kuliev A**, Rechitsky S, Verlinsky O, Ivakhnenko V, Cieslak J, Evsikov S, Wolf G, Angastiniotis M, Kalakoutis G, Strom C, Verlinsky Y. Birth of healthy children after preimplantation diagnosis of thalassemias. *J Assist Reprod Genet* 1999; **16**: 207-211 [PMID: 10224564 DOI: 10.1023/A:1020316924064]

15 **Kanavakis E**, Vrettou C, Palmer G, Tzetis M, Mastrominas M, Traeger-Synodinos J. Preimplantation genetic diagnosis in 10 couples at risk for transmitting beta-thalassaemia major: clinical experience including the initiation of six singleton pregnancies. *Prenat Diagn* 1999; **19**: 1217-1222 [PMID: 10660958]

16 **Rechitsky S**, Kuliev A, Tur-Kaspa I, Morris R, Verlinsky Y. Preimplantation genetic diagnosis with HLA matching. *Reprod Biomed Online* 2004; **9**: 210-221 [PMID: 15333254 DOI: 10.1016/S1472-6483(10)62132-3]

17 **Kuliev A**, Rechitsky S, Verlinsky O, Tur-Kaspa I, Kalakoutis G, Angastiniotis M, Verlinsky Y. Preimplantation diagnosis and HLA typing for haemoglobin disorders. *Reprod Biomed Online* 2005; **11**: 362-370 [PMID: 16176679 DOI: 10.1016/S1472-6483(10)60845-0]

18 **Kuliev A**, Pakhalchuk T, Verlinsky O, Rechitsky S. Preimplantation diagnosis: efficient tool for human leukocyte antigen matched bone marrow transplantation for thalassemia. *Thalassemia Reports* 2011; **1**: e1

19 **Gibson RA**, Morgan NV, Goldstein LH, Pearson IC, Kesterton IP, Foot NJ, Jansen S, Havenga C, Pearson T, de Ravel TJ, Cohn RJ, Marques IM, Dokal I, Roberts I, Marsh J, Ball S, Milner RD, Llerena JC, Samochatova E, Mohan SP, Vasudevan P, Birjandi F, Hajianpour A, Murer-Orlando M, Mathew CG. Novel mutations and polymorphisms in the Fanconi anemia group C gene. *Hum Mutat* 1996; **8**: 140-148 [PMID: 8844212]

20 **Mitsunaga S**, Tokunaga K, Kashiwase K, Akaza T, Tadokoro K, Juji T. A nested PCR-RFLP method for high-resolution typing of HLA-A alleles. *Eur J Immunogenet* 1998; **25**: 15-27 [PMID: 9587741 DOI: 10.1046/j.1365-2370.1998.00093.x]

21 **Blasczyk R**, Hahn U, Wehling J, Huhn D, Salama A. Complete subtyping of the HLA-A locus by sequence-specific amplification followed by direct sequencing or single-strand conformation polymorphism analysis. *Tissue Antigens* 1995; **46**: 86-95 [PMID: 7482512 DOI: 10.1111/j.1399-0039.1995.tb02483.x]

22 **Foissac A**, Salhi M, Cambon-Thomsen A. Microsatellites in the HLA region: 1999 update. *Tissue Antigens* 2000; **55**: 477-509 [PMID: 10902606 DOI: 10.1034/j.1399-0039.2000.550601.x]

23 **Rechitsky S**, Kuliev A. 2010 Novel indications for preimplantation genetic diagnosis. 10th International Congress on Preimplantation Genetic Disgnosis, 5-8 May, Montpellier, France. *Reprod Biomed Online* 2010; **20** (Suppl): S1-2

24 **Gaziev J**, Lucarelli G. Stem cell transplantation for thalassaemia. *Reprod Biomed Online* 2005; **10**: 111-115 [PMID: 15705305]

25 **Kahraman S**, Karlikaya G, Sertyel S, Karadayi H, Findikli N. Clinical aspects of preimplantation genetic diagnosis for single gene disorders combined with HLA typing. *Reprod Biomed Online* 2004; **9**: 529-532 [PMID: 15588472 DOI: 10.1016/S1472-6483(10)61637-9]

26 **Kahraman S**, Beyazyurek C, Ekmekci CG. Seven years of experience of preimplantation HLA typing: a clinical overview of 327 cycles. *Reprod Biomed Online* 2011; **23**: 363-371 [PMID: 21782513 DOI: 10.1016/j.rbmo.2011.05.016]

27 **Kahraman S**. PGD for HLA: Clinical Outcomes of HLA compatible transplantation following PGD. *Reprod Biomed Online* 2013; **26** (Suppl 1): S9-10 [DOI: 10.1016/S1472-6483(13)60030-9]

28 **Verlinsky Y**, Rechitsky S, Sharapova T, Laziuk K, Barsky I, Verlinsky O, Tur-Kaspa I, Kuliev A. Preimplantation diagnosis for immunodeficiencies. *Reprod Biomed Online* 2007; **14**: 214-223 [PMID: 17298726 DOI: 10.1016/S1472-6483(10)60790-0]

29 **Hellani A**, Laugé A, Ozand P, Jaroudi K, Coskun S. Pregnancy after preimplantation genetic diagnosis for Ataxia Telangiectasia. *Mol Hum Reprod* 2002; **8**: 785-788 [PMID: 12149412 DOI: 10.1093/molehr/8.8.785]

30 **Verlinsky Y**, Rechitsky S, Sharapova T, Morris R, Taranissi M, Kuliev A. Preimplantation HLA testing. *JAMA* 2004; **291**: 2079-2085 [PMID: 15126435 DOI: 10.1001/jama.291.17.2079]

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