

Immunohistochemical staining of frozen sections was performed using antibodies for the integrins $\alpha 1\beta 1$, $\alpha 4\beta 1$ and $\alpha v\beta 3$ with the alkaline phosphatase-antialkali phosphatase technique. Immunolabelled sections were analysed semi-quantitatively using the HSCORE method, by a single observer blinded to the groups. The Mann–Whitney *U*-test was used to compare groups.

Results: Results are expressed as median HSCORE: glandular epithelial staining showed increased expression of $\alpha 1\beta 1$ and $\alpha 4\beta 1$ in women with recurrent fetal loss (3.1 and 2.6, respectively) compared to patients with recurrent loss of empty gestation sacs (0.4 and 0.4); $p < 0.0001$ for $\alpha 1\beta 1$ and $p = 0.0006$ for $\alpha 4\beta 1$. There was increased expression of $\alpha 1\beta 1$ and $\alpha 4\beta 1$ in the endometrium of women with recurrent fetal loss when compared to fertile controls but this did not reach statistical significance. There was no difference in glandular staining for $\alpha v\beta 3$ between the groups. There was no significant difference in the median ages among the groups.

Conclusions: Increased expression of $\alpha 1\beta 1$ and $\alpha 4\beta 1$ in women with recurrent fetal loss, and decreased expression in recurrent loss of empty sac may suggest that integrin expression is impaired not only in implantation failure, but also in recurrent early pregnancy loss. Differences in integrin expression in the two recurrent miscarriage groups suggest varying factors and mechanisms in the miscarriage process in fetal loss and loss of empty gestation sac. Further research is needed to ascertain the role of $\alpha 1\beta 1$ and $\alpha 4\beta 1$ in the establishment of early pregnancy.

O-032 The role of complementary therapies in a modern fertility center

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Introduction: The optimal treatment of infertile couples requires a more comprehensive treatment approach than just clomiphene citrate, gonadotropins or assisted reproductive procedures. The success of the patient attempting to conceive may be affected by the patient's psychosocial state. Physical and emotional stress, depression and anxiety may all adversely affect fertility. Complementary approaches to reproductive wellness, such as acupuncture, yoga and mind–body stress reduction techniques have recently been introduced in the field of assisted reproduction, though sporadically. The goals of this study were to evaluate the current use of and interest in complementary therapies by infertile couples to assess the utility of such therapies for attracting and treating infertile patients.

Materials and methods: A brief anonymous questionnaire was completed by 101 consecutive patients actively undergoing fertility treatments at a medium-sized private fertility center in the American Pacific Northwest. The questionnaire contained questions regarding demographics, patients' history of infertility treatments, current use of and interest in participating in complementary therapies designed specifically to support traditional fertility treatments. Information was collected regarding acupuncture, naturopathic medicine, yoga, stress reduction and group support. Respondents were asked if the availability of organized complementary approaches would affect their choice of a fertility center.

Results: The mean age of respondents was 34.9 years old (SD 5.0, range 25–46). The mean duration of infertility was 2.21 years (SD 1.6). A total of 64.3% respondents had completed intrauterine insemination with or without clomiphene citrate and 18.8% had or were currently undergoing *in vitro* fertilization. A total of 78.2% agreed that complementary therapies would improve their experience while undergoing treatment, 19.8% neither agreed nor disagreed (were neutral with regard to the statement) and 2.0% disagreed. Whereas 79.2% respondents agreed that such therapies would improve their chances of success with fertility treatment, 18.8% were neutral and 3.0% disagreed. The most commonly utilized therapy was yoga—37.8% were already doing it and 39.6% were interested in participating. Slightly less, or 34.3%, were under the care of an acupuncturist and 37.8% expressed interest in the treatment. Approximately 16.7% were followed by a naturopathic physician whereas 24.6% expressed interest. Surprisingly, only 8.8% and 6.2% were already doing stress reduction techniques and group therapy respectively but 33.4% and 16.7% were interested in participating in these respective therapies, demonstrating significant unmet need. A total of 67.0% of respondents said that

the presence of complementary therapies would influence their choice of where to seek treatment.

Conclusions: Infertile patients commonly use complementary therapies such as acupuncture, yoga and counseling. There is a significant unmet need for therapies designed to reduce stress and provide group support. The majority of patients said that the availability of complementary services would affect their choice of where to seek treatment. Future research is underway to evaluate the effects of complementary therapies on fertility treatments and potential contribution of a wellness program to a fertility center's success.

FREE COMMUNICATION

Session 07 – Embryology

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10:00–11:30

O-033 Search for epimutations in imprinting center of chromosome 15 in first-trimester spontaneous abortions

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Introduction: There are growing number of reports that have suggested a close link between genomic imprinting defects and assisted reproductive technologies (ARTs). Imprinting is an epigenetic phenomenon related to differential expression of maternal and paternal genes. The parental copies of imprinted regions differ with respect to DNA methylation and histone modification. Imprinted genes are not randomly distributed in the human genome, but tend to occur in clusters. This suggests that the primary control of imprinting is not at the single gene level, but at the chromosome domain level. Indeed, several clusters (15q11–q13 and 11p15.5 regions) have been found to contain a *cis*-acting imprinting centre (IC) which controls imprint establishment during gametogenesis and maintenance in somatic cells. Several mechanisms are responsible for imprinting disorders, such as chromosome microdeletions, uniparental disomies, mutations in imprinted genes and epimutations of the IC. These aberrations lead to defined clinical syndromes and probably affect embryo development. Surprisingly, the hypomethylation of the IC regions 15q11–q13 and 11p15.5 on the maternal chromosomes were found in children with Angelman or Beckwith–Wiedemann syndromes born after ART. At the same time, the prevalence as well as possible selective disadvantages of the IC epimutations during embryo development in non-ART pregnancies are still unknown. In order to determine this effect, the DNA methylation screening of the IC of chromosome 15 (*SNURF-SNRPN*) in human spontaneous abortions has been initiated. There are some evidences, which suggested maternal methylation imprints establishment on the chromosome 15 during or after fertilization and blastomeres cleavage (1). It is possible that this process is a subject for aberrant chromatin changes at the time of epigenetic genome reprogramming in embryo somatic cells.

Methods: Forty first-trimester spontaneous abortions with normal karyotype and eight induced abortions as a control group were investigated. DNA was isolated from extraembryonic mesoderm and cytotrophoblast by standard proteinase K digestion and phenol–chloroform extraction. Bisulfite DNA conversion and methylation-specific PCR of the CpG-island in *SNURF-SNRPN* were performed by standard protocols (2).

Results: Two PCR products were detected in all studied embryos. One of the products was specific to maternal methylated allele, and other was specific to paternal non-methylated locus. This result is a feature of normal individuals. Evidently, no cases of aberrant IC methylation in the extraembryonic tissues of spontaneous abortions with different patterns of epigenetic reprogramming were observed.

Conclusions: Our data indicate a normal DNA methylation status of the *SNURF-SNRPN* in spontaneous abortions after non-ART pregnancies. Probably, disruption of genomic imprinting in chromosome 15 cluster has no a crucial effect for human embryogenesis. However, further analysis of other imprinted loci is important for delineation of the pathogenetic role of the IC's epimutations in early embryo development. The accumulation of such a dataset

should have some significance for the estimation of the epigenetic risks related to ART.

References

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O-034 The DNA methylation and histone acetylation of oocytes in juvenile and adult mice

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Introduction: Before the resumption of meiosis, the oocyte must accumulate sufficient maternal transcripts to support subsequent meiotic divisions and early embryo development. Epigenetic modification is the key mechanism to regulate the dynamic transcriptional activity of oocyte genome during its growth. However, the timing and mechanisms of genome-wide epigenetic modification are poorly understood during its growth. Therefore, our objective was to investigate the specific changes of DNA methylation and histone acetylation undergone by chromatin remodeling during oocyte growth. To investigate epigenetic changes, DNA methylation and histone acetylation were examined in growing, and in maturing oocytes from juvenile and adult mouse ovaries.

Materials and methods: Growing oocytes of juvenile mice were retrieved from ovaries of 5, 10, 15 and 20 days old. Oocytes of adult mice were harvested from CD-1 mice aged 8–12 weeks. To obtain follicles within ovaries, the dissected ovaries were digested by incubating in 2.0 mg/ml collagenase at 37°C for 15–30 min followed by vigorous vortexes. After being denuded of cumulus cells, the oocytes were sorted into five groups based on oocyte diameter: (i) >75 µm (fully grown); (ii) 60–70 µm; (iii) 50–59 µm; (iv) 40–49 µm; and (v) 20–39 µm. In order to visualize histone acetylation and DNA methylation, the oocytes were fixed and stained with two antibodies: TRITC-conjugated anti-acetylated histone H3 (at lysine 18), and FITC-conjugated anti-5'-methyl-Cytosine, respectively, using laser scanning confocal microscopy.

Results: A total of 442 oocytes were included in the present study. It was observed that DNA methylation had been acquired during oocyte growth in accordance with the developmental age. The DNA methylation originating from several discrete foci was present in the early growing oocyte genome, which was overlapped with pericentric heterochromatin regions. The intensity of overall DNA methylation gradually increased and extended along with the oocyte growth until it reached to the fully grown stage, at which time intense signals of DNA methylation and histone acetylation were colocalized. On the other hand, the removal of histone acetylation occurred during the resumption of meiosis when the chromosomes were in a condensed state. Together, genome-wide epigenetic modifications were steadily acquired during oocyte growth, until the later stages of its growth and maturation.

Conclusions: The results of this study demonstrate that temporal and spatial changes in DNA methylation and histone acetylation are highly dynamic and occur in specific patterns throughout oocyte growth and maturation. The genome-wide epigenetic changes (DNA methylation and histone acetylation) were steadily acquired during oocyte growth until the concluding stages of growth and maturation. It is suggestive that those epigenetic modifications with temporal and spatial patterns are imperative to the completion of oocyte meiosis and the subsequent embryo development. These findings have far reaching implications for assisted reproductive technologies which rely upon immature and growing oocytes.

O-035 Expression of GCN5 and HDAC1 in early mouse parthenogenetic embryo

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Introduction: Parthenogenesis is a means of producing descendants solely from maternal germ cells. Accumulating evidences have shown that the

expression of a group of imprinted genes has undergone great changes in parthenogenetic embryo. Histone acetylation is one of the key mechanisms involving the procedures of gene imprinting. Both histone acetyltransferase general control of nucleotide acid synthesis (GCN5) and histone deacetylase 1 (HDAC1) are enzymes which form a complicated complex to altering the levels of histone acetylation in chromatin. Normally, GCN5 is considered as transcriptional coactivator, whereas HDAC1 is considered as transcriptional repressors. To investigate the changes in expression and localization of GCN5 and HDAC1 in early mouse parthenogenetic embryo, the following study has been carried out.

Materials and methods: The mouse oocytes were collected at post human chorionic gonadotrophin (hCG) 16–18 h, and hyaluronidase was used to remove mucous cumulus cells to retrieve nude oocytes. The nude oocytes were treated with 5 µM calcium ionophore A23187 for 5 min followed by 25 µM 6-dimethylaminopurine for 4 h. Parthenogenetic embryo at the stages of one-cell, two-cell and four-cell were picked up at 4–6 h, 24–26 h and 48–50 h after parthenogenetic activation, respectively. Meanwhile, the mouse embryos at the stages of one-cell, two-cell and four-cell through standard *in vitro* fertilization (IVF) were picked up at 4–6 h, 24–26 h and 48–50 h after IVF. Fluorescent immunocytochemistry was used to determine the localizations and intensities of the expressions of GCN5, HDAC1 and proliferating cell nuclear antigen (PCNA) in the parthenogenetic and IVF embryos at the stages of one-cell, two-cell and four-cell, respectively.

Result: (i) In IVF group, the expression of GCN5 was observed in the cytoplasm and nuclei at the stages of one-cell and two-cell, and only in the cytoplasm of four-cell embryos. Meanwhile, the obvious expression of HDAC1 was seen only in the nuclei of the embryos at all stages and the intensities of immunofluorescence were duplicated with the increase in cell number of embryos. (ii) In parthenogenesis group, the expression patterns of GCN5 and HDAC1 were similar to those of IVF group. However, their expression intensities were significantly reduced compared with IVF. (iii) However, no matter in IVF and parthenogenesis or in the different stages the expression of PCNA as a proliferative marker during embryonic development remained no obvious changes.

Conclusions: There might be decrease in the expression levels of GCN5 and HDAC1 without the change of PCNA in mouse early parthenogenetic embryos in this study. The results suggested that the changes of some important gene expressions during mouse early parthenogenetic embryos' development could be regulated by histone acetylation but not deduced by the defection of proliferation.

O-036 Epigenetics and cytogenetics mechanisms for chromosomal mosaicism arising in human embryos

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Introduction: Progress in preimplantation genetic diagnosis as well as introduction of molecular cytogenetic techniques into analysis of human reproductive wastages have revealed a high frequency of chromosomal mosaicism. However, the molecular mechanisms leading to aberrant chromosome segregation in embryo cells remain illusive. Current opinions in developmental genetics suggest the cross-related role of genetic as well as epigenetic factors in the regulation of fetal growth. The key events in early embryogenesis, which have a great significance for further tissue-specific patterns of mosaic cell lines compartmentalization, are blastomeres cleavage and differentiation of trophectoderm (TE) and inner cell mass (ICM). Significantly, these stages of development concur with epigenetic genome reprogramming. It is interesting to note, that increasing of mosaic karyotypes in cleavage embryos is observed during global genome demethylation as well as cell-cycle checkpoints inactivation. It is possible that the loss of epigenetic control of the genome stability and errors in chromosome segregation are closely related.

Materials and methods: Literature data about origin (meiotic or mitotic) as well as distribution of aneuploid cells in the TE and ICM derivatives of spontaneous abortions were revised. A mathematical model for tissue-specific mosaic cell lines compartmentalization has been developed. The tissue-specific patterns of chromosomal mosaicism in 63 spontaneous abortions were investigated by interphase FISH of native extraembryonic tissues with centromere-specific DNA probes. DNA promoter methylation of the cell-cycle G₁-checkpoint genes (*CDKN2A* and *RBI*) was analysed by methyl-specific

PCR in 11 first-trimester spontaneous abortions with mosaic aneuploidy in the cytotrophoblast (TE derivative) and extraembryonic mesoderm (ICM derivative) as well as in the group of 23 pregnancy term-related induced abortions.

Results: According to the model proposed the distribution of chromosomal mosaicism in spontaneous abortions is related to the timing of mitotic errors (before or after TE and ICM differentiations) as well as with the risk of meiotic or mitotic non-disjunction in the etiology of aneuploidy. It is shown that the presence of aneuploid cells in the cytotrophoblast indicates the meiotic errors. Otherwise, the localization of mosaic cells in the extraembryonic mesoderm solely is a result of mitotic non-disjunction. Finally, the presence of mosaicism both in TE and ICM derivatives could be related to meiotic or mitotic errors in the genesis of aneuploidy.

Experimental results have revealed three groups of abortions with different patterns of abnormal cell lines compartmentalization in the extraembryonic tissues. The frequency of abortions with preferred localization of aneuploid cells in the extraembryonic mesoderm or in the cytotrophoblast was 42% and 19%, respectively. The rate of embryos with uniform distribution of abnormal cell lines between two tissue types was 39%. Hypermethylation of the *CDKN2A* and *RBI* promoters was found in the extraembryonic mesoderm of 91% and 88% spontaneous abortions. The corresponding values in the cytotrophoblast were of 82% and 56%. None of the mosaic embryos without hypermethylation of one or two genes was found. At the same time, no cases of aberrant methylation were found in the placental tissues of pregnancy term-related 23 induced abortions.

Conclusions: The results of molecular cytogenetics analysis provide experimental evidence for a high rate of mitotic errors in the etiology of mosaic aneuploidy in spontaneous abortions. At the first time an aberrant hypermethylation of the G1-checkpoints genes in mosaic embryos has been described. Our findings point out a link between aberrant epigenetic reprogramming and genome instability in the etiology of chromosomal mosaicism. In the light of current discussion about epigenetic risks of assisted reproductive technologies, the presented results indicate one of the possible pathways for mosaicism arising and should be taken into account for improving the preimplantation aneuploidy screening.

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O-037 Pronuclear morphology in zygotes generated by chromosomally normal oocytes

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Introduction: The complex series of events occurring in the oocytes after fertilization play a key role in the design of the embryonic axis. The correct sequence and coordination of the processes involved in this phase represent a basic step for cell determination in the developing embryo. It has been reported that pronuclear morphology is an indicator of these steps, and that their scoring could assist in the prediction of embryo development and chromosomal complement. In the present study, the inseminated oocytes had been classified as chromosomally normal on the basis of first polar body analysis. The morphology of pronuclear zygotes was evaluated and related to the configurations which, based on the chromosomal analysis performed in preimplantation embryos in previous studies, were assessed as predictive of euploidy.

Materials and methods: Between March 2004 and November 2005, 272 patients with a mean maternal age of 37.5 ± 4.2 years underwent 308 assisted conception cycles in combination with the screening of aneuploidy on first polar body. At ~1 h after collection, oocytes were denuded by hyaluronidase treatment and polar body biopsy on 618 MII oocytes started immediately. The chromosomes 13, 15, 16, 18, 21 and 22 were studied by using FISH and euploid oocytes were inseminated up to a maximum of three per patients, as established by the current Italian legislation on IVF. At 16 h after insemination, oocytes were checked for the presence and morphology of pronuclei, nucleoli and polar bodies.

Results: When analyzing the position of pronuclei, 88% of zygotes had juxtaposed pronuclei (83% for juxtaposed centralized pronuclei and 5% for juxtaposed non-centralized pronuclei); for the size and distribution of nucleoli, the majority of zygotes had large-size aligned (29%) or non-aligned (39%)

nucleoli; the position of the second polar body was in the longitudinal axis of pronuclei in 48% of cases and in the perpendicular axis in 46% of zygotes. The combination of these patterns demonstrated that 80% of zygotes had the configurations with centralized juxtaposed pronuclei, large-size aligned or scattered nucleoli and polar bodies located in the longitudinal or perpendicular axis of pronuclei. Accordingly, zygotes with these configurations gave rise to 13 gestational sacs out of 15 for which a correlation with implantation was established, and to 81% of zygotes which were possibly involved in implantation.

Conclusions: These results are in full agreement with those derived from the FISH analysis of preimplantation embryos, in which the highest proportion of chromosomally normal embryos belonged to the same configurations identified by the euploid oocytes in the present study. These observations confirm that some patterns of pronuclear morphology are associated with a higher proportion of euploidy. Beside reaffirming the relevance of the oocyte quality in determining its fate, the current results support the strength of this scoring system for the prediction of zygote viability.

O-038 Bromodomain protein BRD4 in mouse oocytes and early embryos: its possible involvement in zygotic gene activation and reprogramming of gene expression

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Introduction: A mammalian nuclear protein BRD4 carries two bromodomains, evolutionarily conserved protein modules. It tightly associates with mitotic chromosomes through binding to the acetylated histone H3 and H4, suggesting its possible role in the transmission of relevant information across cell division. Indeed, mouse embryos nullizygous for BRD4 die shortly after implantation. To obtain a clue to the molecular mechanism, we investigated the spatio-temporal behaviors of BRD4 in conjunction with histone acetylation during mouse oocyte maturation and fertilization.

Materials and methods: Oocytes were collected from superovulated immature BDF1 and cultured in TYH medium to obtain GV-, GVBD-, MI- and MII-stage oocytes in the presence or absence of trichostatin A (TSA), an inhibitor of histone deacetylases. Embryos at the 1-cell, 2-cell and 2-cell metaphase stages were prepared by *in vitro* fertilization. The expression levels of BRD4 in oocytes and early embryos were determined by RT-PCR and Western blotting. The cellular distributions of BRD4 and acetylated histone H3 and H4 were investigated by immunofluorescence using antibodies against BRD4 and individual acetylated histones. To further examine the distribution of BRD4 during meiosis, the mRNA of enhanced green fluorescent protein-fused BRD4 (EGFP-BRD4) was prepared and microinjected into the cytoplasm of GV-stage oocytes. The microinjected oocytes were incubated in the presence or absence of TSA and then collected at GVBD-, MI- and MII-stages.

Results: The expression levels of BRD4 mRNA and protein were gradually increased toward the end of the second meiosis. Immunofluorescence revealed that BRD4 was preferentially localized in the nucleus and/or the condensed chromosomes of GV-stage oocytes and embryos at the 1-cell, 2-cell and 2-cell metaphase stages. In contrast, not only endogenous BRD4 but also overexpressed EGFP-BRD4 were dispersed into the cytoplasm of GVBD-, MI- and MII-stage oocytes, whose histones H3 and H4 became hypoacetylated. Treatment of these oocytes with TSA dramatically induced the hyperacetylation of the histones H3 and H4 on their condensed chromosomes; however, endogenous BRD4 remained to be dissociated from the chromosomes. Chromosomal localization of BRD4 in GVBD and meiotic oocytes could only be achieved by BRD4 overexpression together with histone hyperacetylation induced by co-treatment with TSA.

Conclusions: Our results suggest that there may be possible mechanism(s) for active dislodgment of BRD4 from the meiotic chromosomes and its re-localization to the mitotic chromosomes, which, together with upregulation of BRD4, may participate in epigenetic regulation of gene expression such as reprogramming and zygotic gene activation.