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Anaylsis of spare embryos following PGD/PGS

Follow-up data and how it can help guide your laboratory practices

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Overview

- Why perform follow-up
- FISH-based follow-up
- PCR-based follow-up
- Practical experience with an “underperforming” test
- Confirmatory FISH-reducing false positives

Why should your lab perform follow-up?

- Follow-up of untransferred embryos following PGD can help the lab:
  - Catch mistakes made during testing that could lead to a misdiagnosis
  - Learn about false-positive and false-negative results and how often they occur in the lab
  - Learn about mosaicism in early embryos and how it affects diagnosis, especially in aneuploidy screening
  - Manage underperforming lab tests and show areas that need improvement
  - Determine an internal error rate which should be available to patients at initial consultation

Follow-up of untransferred embryos

- Donating embryos to research
  - IRB approved program to systematically collect embryos for future research and our follow-up program
  - Specific consent that spells out the options to the patient for untransferred embryos
    - Disposal
    - Research
    - Donate to another couple (only normal embryos)
  - Genetic counselor speaks to each patient about untransferred embryos and coordinates receipt of embryos into research program, including follow-up

FISH-based follow-up testing

- Follow-up of untransferred embryos donated by couple to "research"
  - Our protocol is to choose at least one embryo that…
    - Has continued to progress following embryo biopsy
    - Had a FISH result on day-3
    - Preferentially choose embryos that had a single abnormality
    - Will spread "normal" embryos if they are donated for research to look at false-negative rate

FISH-based follow-up testing

- Steps
  - Remove zona pelucida using pronase
  - When zona is almost fully digested, the embryo is rinsed in 5% FBS/dPBS then placed on slide within etched area
  - Embryo is placed in 12.5 ul of Tween 20/HCl
  - Drops of Tween/HCl are added while visualizing blastocyst to dissociate the cells and lyse the cell membranes
  - Slide is allowed to air dry
  - Slide is fixed in Formalin followed by dehydration in sequential ethanol
  - Cells are located and mapped prior to addition of FISH probes
  - Follow-up embryos are always probed with the same mix as the original PGD testing

FISH-based follow-up testing

- Concordance and non-concordance
  - Our definition is as follows
    - Concordant embryo scores are those where the embryo score on day-3 matches the embryo score on day-5 following whole embryo spreading followed by FISH
    - Our definition allows for “different” abnormalities between day-3 and day-5 to be concordant
    - We also note “perfect” concordance in our data collection when the day-3 and day-5 scores match exactly

FISH-based follow-up testing

- Reporting
  - We prepare a report for each case that is followed-up that describes the testing performed, and details the results from day-3 and day-5
  - This report is placed in the chart with the original case
  - Our goal is to follow-up at least one embryo from each case biopsied at our center
  - All embryos that are followed-up are collected in a database that keeps track of concordance/non-concordance, embryos with no results and detailed data comparing each embryo
  - We have performed a research study with a collaborator (transport PGD) to follow up untransferred embryos but have not looked at the data yet

PCR-based follow-up testing

- Follow-up of untransferred embryos donated by couple to “research”
  - Our protocol is to follow-up as many embryos as possible, but only embryos that…
    - Have continued to progress following embryo biopsy
    - Had a PCR result on day-3

PCR-based follow-up testing

- Steps
  - Remove zona pelucida using pronase
  - Place whole embryo into PCR tube containing 2.5 ul of lysis mix (NaOH/DTT)
  - Cover with 2 drops of oil
  - Place in hotblock at 65°C for 10 minutes
  - Place samples on ice until all are collected
  - Add MDA master mix or PCR mix
  - Amplify DNA with same primers used in original testing

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PCR-based follow-up testing

- Concordance and non-concordance
  - Our definition is as follows
    - Concordant embryo scores are those where the embryo score on day-3 matches the embryo score on day-5
- Reporting
  - We prepare a report for each case that describes the testing performed, and details the results from day-3 and day-5
  - This report is placed in the chart with the original case
  - Our goal is to follow-up at least one embryo from each case biopsied at our center
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Practical experience with an "underperforming" test

- Original test developed and validated on lymphocytes and blastomeres
- Probe set included
  - CEP X (Xp11.1-q11.1, DXZ1)
  - CEP Y (Yq12, DYZ1)
  - 21q subtelomere (D21S1575)
- Test offered to patients to determine gender of embryos at PGD as well as screen for aneuploidy of chromosome 21

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Practical experience with an “underperforming” test

- Follow-up of untransferred embryos

	“Original” Test	Percentage
Concordant	**7**	**50.0%**
Discordant	**7**	**50.0%**
Undetermined	**0**	
Total Spread	**14**	**100%**

Practical experience with an “underperforming” test

- “New” test developed and validated on lymphocytes and blastomeres
- Probe set included
  - CEP X (Xp11.1-q11.1, DXZ1)
  - CEP Y (Yq12, DYZ1)
  - LSI21 (D21S259, D21S341, D21S342)
- Test offered to patients to determine gender of embryos at PGD as well as screen for aneuploidy of chromosome 21

Practical experience with an “underperforming” test

- Follow-up of untransferred embryos

	“New” Test	Percentage
Concordant	**9**	**60.0%**
Discordant	**6**	**40.0%**
Undetermined	**3**	
Total Spread	**18**	**100%**

Practical experience with an “underperforming” test

- Original test was clearly “underperforming” with a large number of false-positive results
- The issue was clearly a split-signal problem
- Technologists suspected that the true result should have been “normal” but could not, with confidence, score the cells as “normal” for chromosome 21.
- Validated a new test with a different chromosome 21 probe
- Encountered the same problem clinically-test has been discontinued

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Confirmatory FISH

- Any nucleus that contains a single aneuploidy (monosomy or trisomy) will be hybridized with a special second round mix which includes a different probe for the chromosome in question to either confirm or overturn the original score

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Confirmatory FISH

- As a way to combat false-positive results, we are developing tests to “confirm” results of FISH-based tests before the final report is issued
- This will allow us to confirm single aneuploidies seen by FISH with a second probe that localizes to a different region of the chromosome prior to embryo transfer
- Should cut down on false-positive results and allow transfer of more “normal” embryos

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Confirmatory FISH

- 8-probe Aneuploidy Screening
  - Vysis PB kit followed by home-brew X, Y, 15
  - Any probe in the first round can be “confirmed” in the second round
    - i.e. if we see monosomy 16 in the first round on a particular nucleus, the second round mix will be X, Y, 15, 16*
  - Each nucleus on a slide (we spread as many as 8 on a slide) can be probed with a different second round mix
    - i.e. position A was scored as monosomy 16 and will be probed with X, Y, 15, 16*, while position E was scored as trisomy 21 and will be probed with X, Y, 15, 21* at the same time

Confirmatory FISH

- 5-probe Aneuploidy Screening
  - Vysis PGT kit
  - Does not use a second hybridization routinely
  - Will add a second round hybridization so that any probe in the first round can be “confirmed” in the second round
    - i.e. if we see monosomy 18 in the first round on a particular nucleus, the second round mix will be 18*
  - Each nucleus on a slide (we spread as many as 8 on a slide) can be probed with a different second round mix
    - i.e. position A was scored as monosomy 18 and will be probed with 18*, while position E was scored as trisomy 21 and will be probed with 21* at the same time

Confirmatory FISH

- Have worked out probe mixes and washing conditions first using lymphocytes
- Continued development of conditions on day-3 blastomeres from embryos donated for research
  - Each blastomere located and scored for first round mix
  - First round probes stripped using our validated probe strip protocol
  - Second round hybridization with mix of X, Y, 15 and 1 of “confirmation” probes as listed previously
  - Embryos were of unknown aneuploidy status so we only scored for signal intensity of confirmation probe and other included probes

Confirmatory FISH

- Current status
  - Selectively performing confirmatory FISH on clinical embryos that have a single abnormality **without** reporting the results of the confirmation step
  - Selective follow-up of untransferred embryos that underwent confirmatory FISH analysis on day-5 or 6
  - All data from follow-up of these embryos will be kept in a dedicated database to assess the results
    - Original cell score
    - Confirmatory cell score
    - Follow-up embryo score

Confirmatory FISH

- Current Status and the future
  - The lab will continue to analyze the data on the number of confirmations/overturning of original results
  - Will use follow-up to determine the best way to implement confirmation FISH into clinical practice
  - Will continue to make adjustments to lab procedures to reduce or eliminate false-positive and false-negative results

Thank you…

- Local organizing committee
- ESHRE
- Genetics & IVF Institute
  - PGD lab
  - Embryology lab
  - Clinical genetics staff and genetic counselors