DIFFERENTIATION/CULTURE OF EMBRYOID BODIES

<u>Stage 1</u>: 4 days, culture of ES cells on non-coated TC surface in depleted medium

- Seed two times $2.5.10^6$ ES cells in 2x 6 cm TC dishes (non-gelatin-coated) in 2x 5 ml depleted medium (DM).
- Refresh medium every day.
- After 3 days of culture loosened parts of ES colonies which piled up the days before will float in medium; to prevent destruction and loss of these cell clumps refresh cells as follows:
  . transfer medium with wide bore 10 ml pipette to 15 ml conical tube.
  . let sediment ES cell clumps for 1 min.
  . aspirate supernatant carefully, leave some medium.
  . add fresh 5 ml DM, resuspend pellet carefully again with wide bore pipette and seed cell clumps in same dish.
- On this same day prepare agarose layers for stage 2 (next day):
  . autoclave 2% agarose in PBS.
  . wet surface of 2x 3 10 cm TC dishes with ± 5 ml warm agarose solution each.
  . remove air bubbles; leave 10' RT to get the thin layers.
  . equilibrate each agarose layer ON (at least 4 hours) in the incubator with ±10 ml DM.

<u>Stage 2</u>: 6 days, culture on agarose layer in depleted medium

- Collect cell clumps of each of both dishes:
  . aspirate sup with wide bore pipette, transfer to 15 ml conical tube.
  . direct a jet of 5 ml DM across the surface of the dish to dislodge small cell clumps and transfer medium with loosened cell clumps to another conical tube; repeat this until the surface looks empty.
  . let cell clumps sediment for 5' at RT.
  . aspirate sup (carefully, pellet is very loosely packed).
- Aspirate medium of agarose coated dishes.
- Resuspend cell clumps in 20-30 ml DM and spread them over 2-3 agarose coated dishes: incubate untouched for 72 hrs.
- Grown cell clumps form embryoid bodies (EB) which do not attach to the agarose layer.
- After 3 days EB float in medium, refresh (aspirate medium plus EB, let sediment for 5' at RT, aspirate most of the sup, resuspend EB in ± 10 ml DM per 10 cm dish and transfer to same agarose layer) and leave 3 days in incubator.
- In these days differentiated tissues can be seen in EB, after these three days (= after 10 days of culture) beating of heart tissue can be seen.

<u>Stage 3</u>: 4-5 weeks, culture on non-coated TC surface in complete medium

- Collect EB like before, but:
  . Resuspend EB in CM.

. Depending on the number of EB, reseed on 2 - 4 x 6 cm TC dishes (not gelatin coated) with EB of 1 x 10 cm agarose coated petri dish (5 ml/6 cm petri dish).

- Leave dishes 2 days in incubator untouched to allow attachment of aggregates to the dishes (aggregates unattached after 2 days are unlikely to do so).
- Refresh medium of attached EB (thereby removing unattached ones).
- Change medium when necessary (approx. every 2 days) and observe the progress of differentiation over a period of 4-5 weeks.