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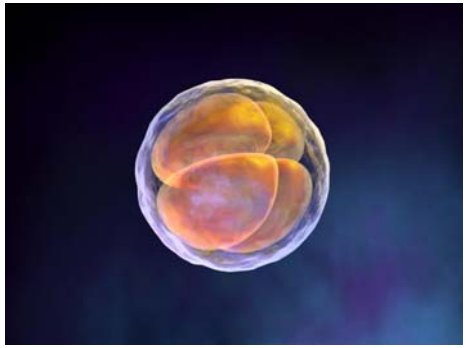
Optimizing Human Embryo Care



STRESS AND HUMAN EMBRYO CULTURE

Dr. Catello Scarica

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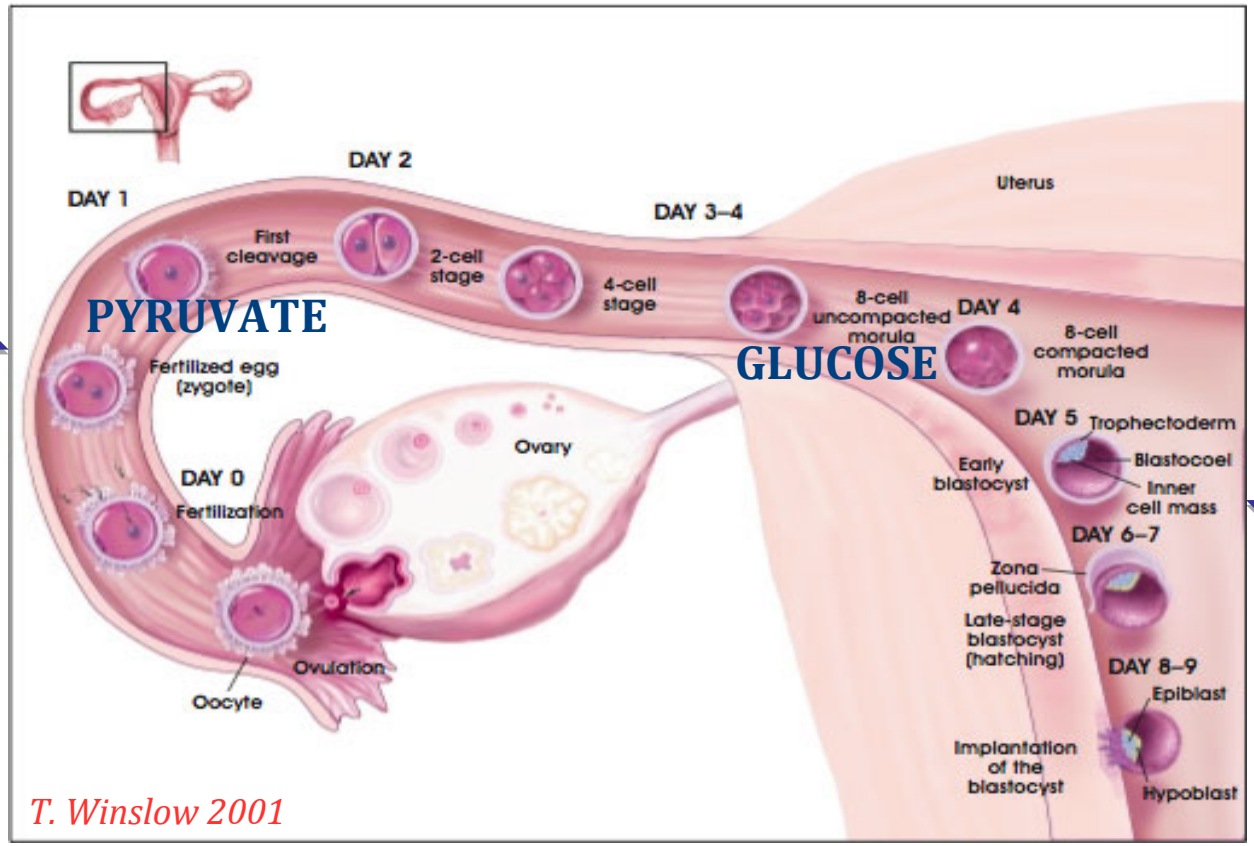
In Vivo Physiology

The in vivo physiologic environment of the early embryo is tightly regulated



O_2 7%

O_2 2%

The environment in which the embryo develops can alter its metabolism, epigenetic marks, and developmental capacity

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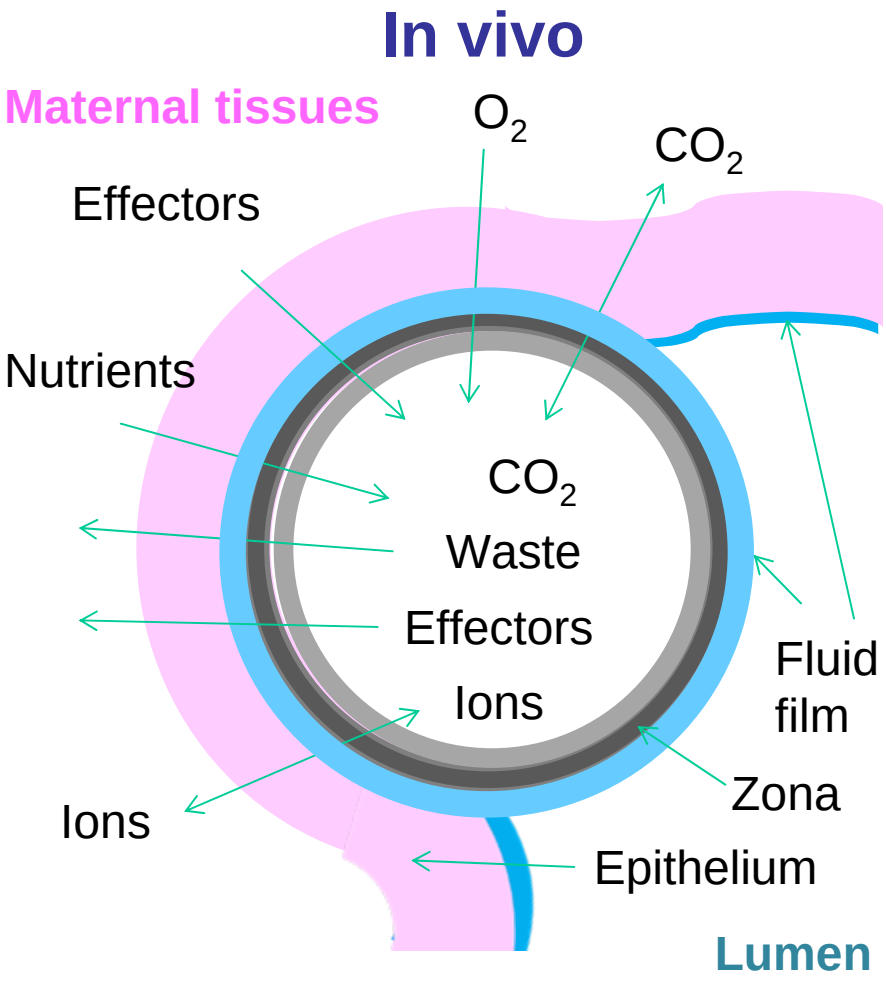
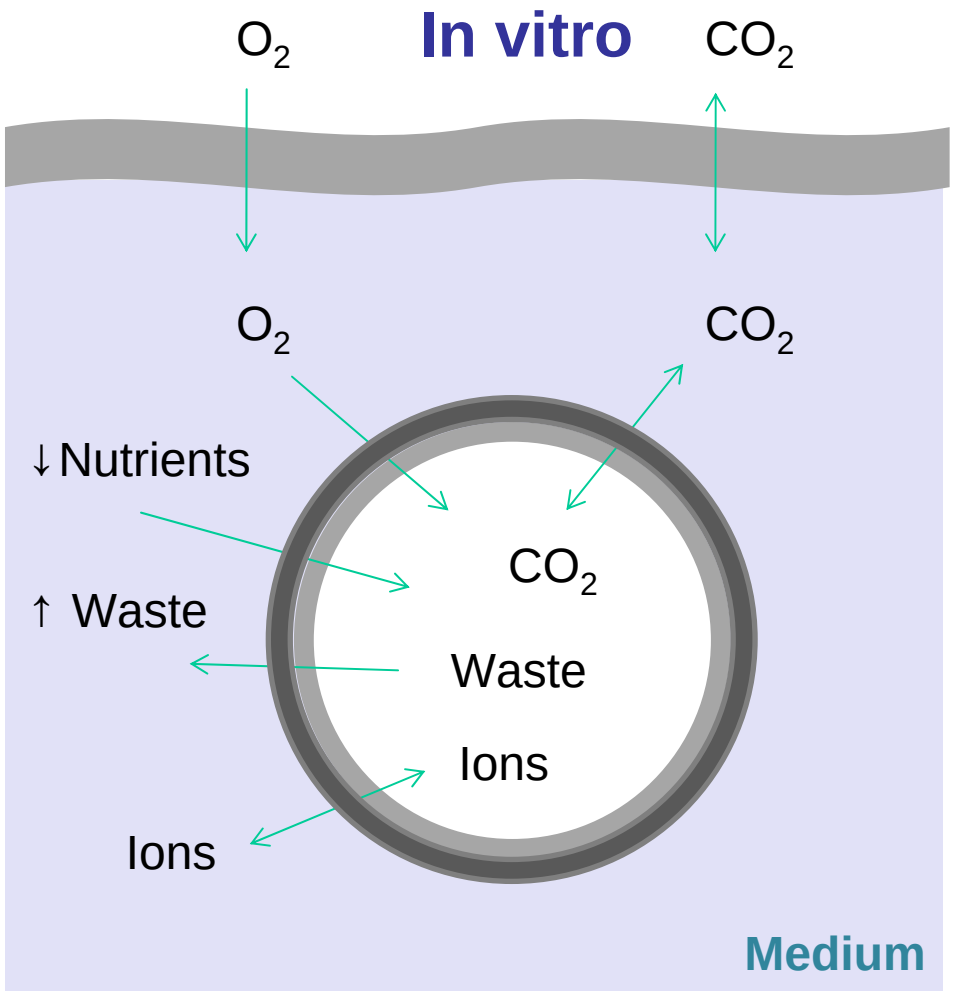
Preimplantation environment

Within the reproductive tract the embryo is likely to be exposed to autocrine, paracrine and endocrine mediators.

By definition, paracrine and endocrine embryotrophic mediators are excluded from culture in simple defined medium





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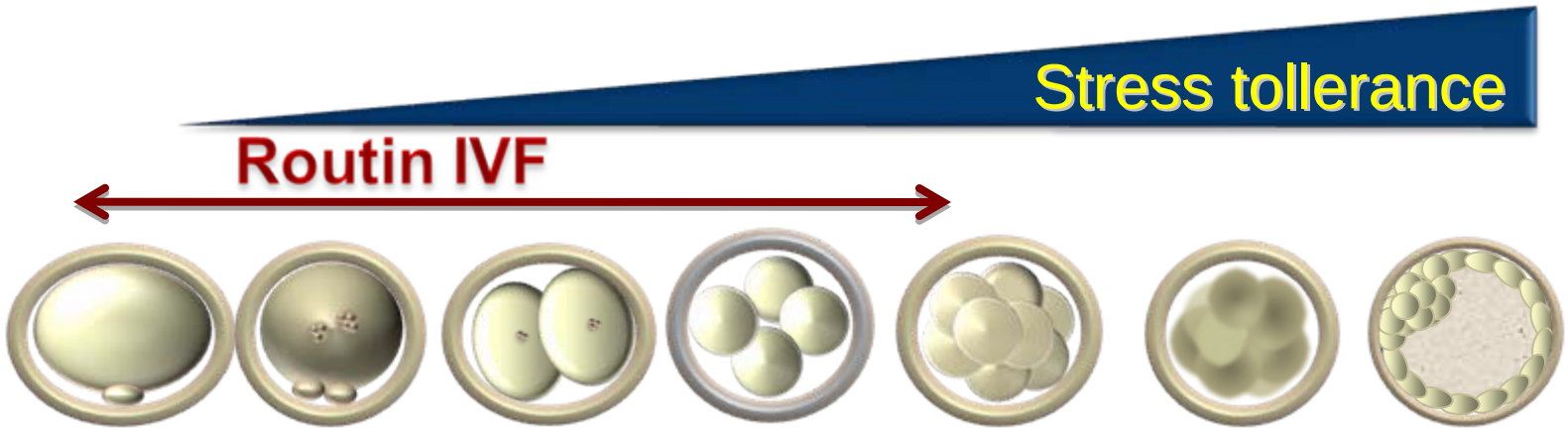
Preimplantation embryo is a free-living organism

The ability of an embryo to respond to changes in its environment is limited during the first cleavage divisions, when most of the embryonic genome is still inactive and when systems for regulating osmotic balance are not completely functional.



Critical period determining blastocyst yeld

Critical period determining blastocyst quality

- Epigenetic remodelling of PN
- First cleavage division
- Embryonic genome activation
- Morula compaction
- Blastocyst formation

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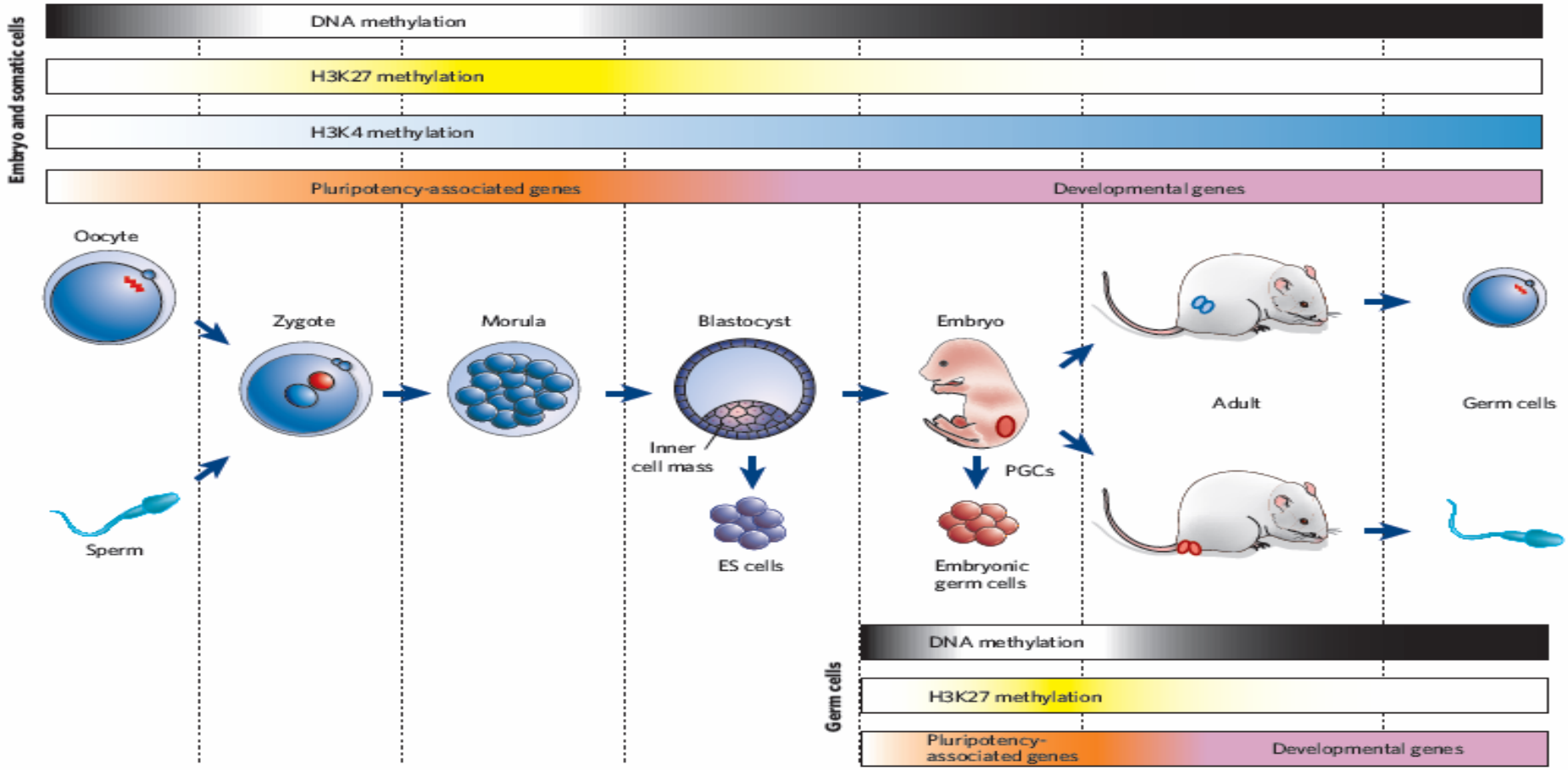
Genotype to phenotype and the journey of reproductive cells

“Development is, by definition, epigenetic”



Review: Epigenetic

- Epigenetics: process that regulates gene activity without affecting the genetic (DNA) code and is heritable through cell division

- Epigenotype: chromatin structure modifications depending in large part on:

  Histone modifications (mediated by covalent modification on histone tails, flexible and reversible, mediate short term effect on gene expression)

  Methylation of cytosines within CpG dinucleotides*

  - **Gene silencing mark mediating monoallelic gene espression for a subset of genes (imprinted loci)**
  - **Modification is very stable (but is reversible)**
  - **Mediate long term transgenerational control of gene expression**

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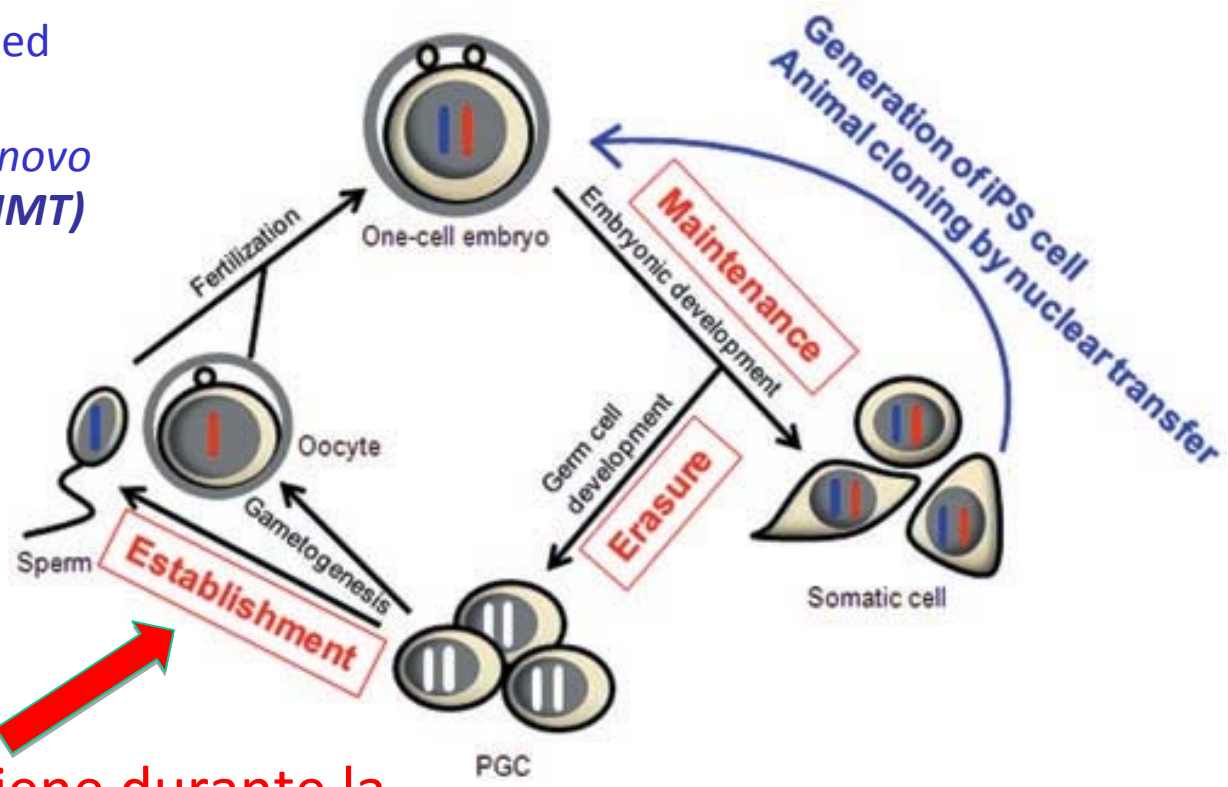
Life cycle of the genomic imprinting

DNA methylation is introduced during either oogenesis or spermatogenesis, by the *de novo* ***DNA methyltransferase (DNMT)***



L'IMPRINTING avviene durante la **GAMETOGENESI**

Yufeng Li, Hiroyuki Sasaki 2011

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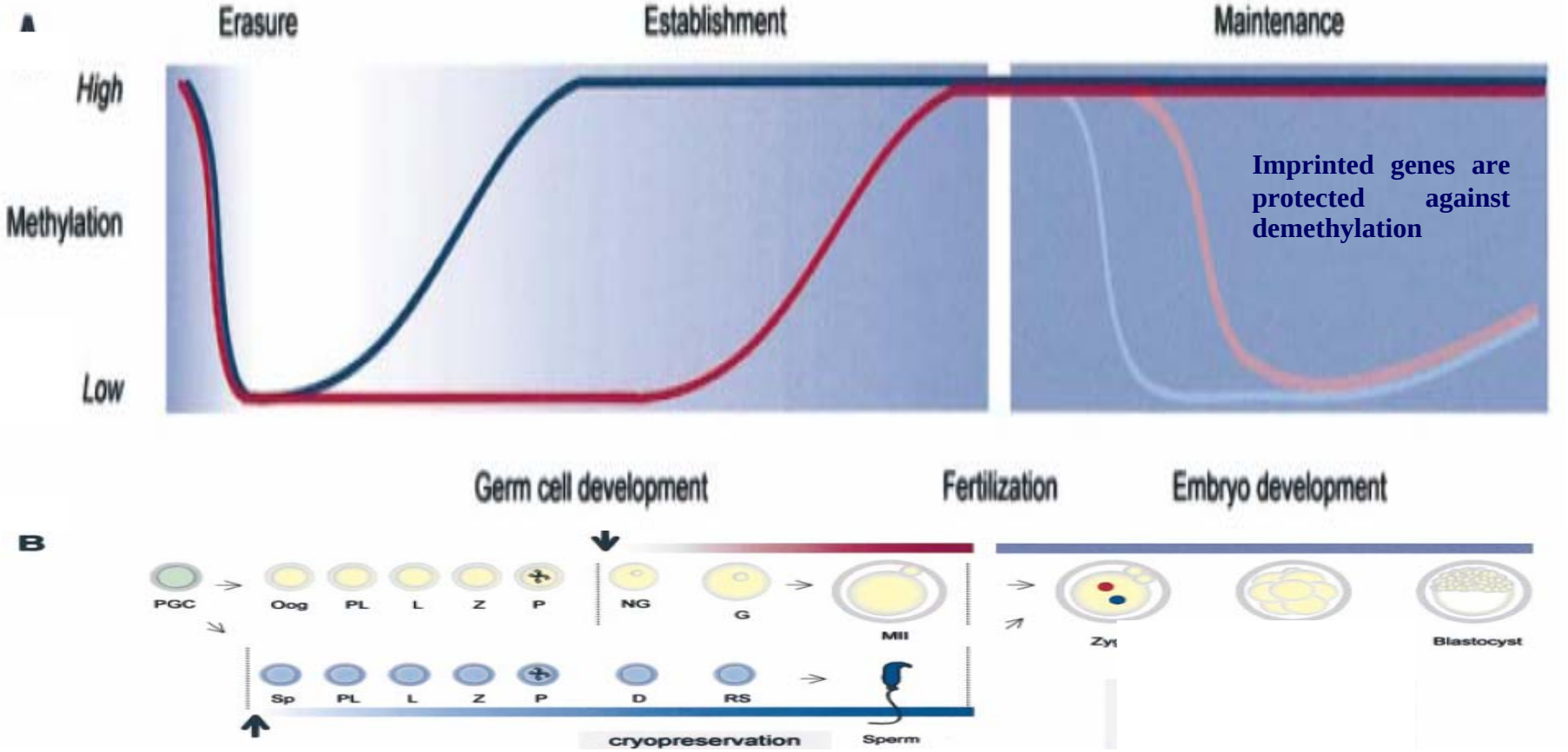
Cycle of gene imprinting and IVF procedures

Germ cell development and early embryogenesis are crucial windows in the erasure, acquisition and maintenance of epigenetic marks. Imprints established in the gametes must be faithfully maintained during preimplantation development while the methylation status of non-imprinted genes undergoes dynamic changes (transgenerational inheritance)



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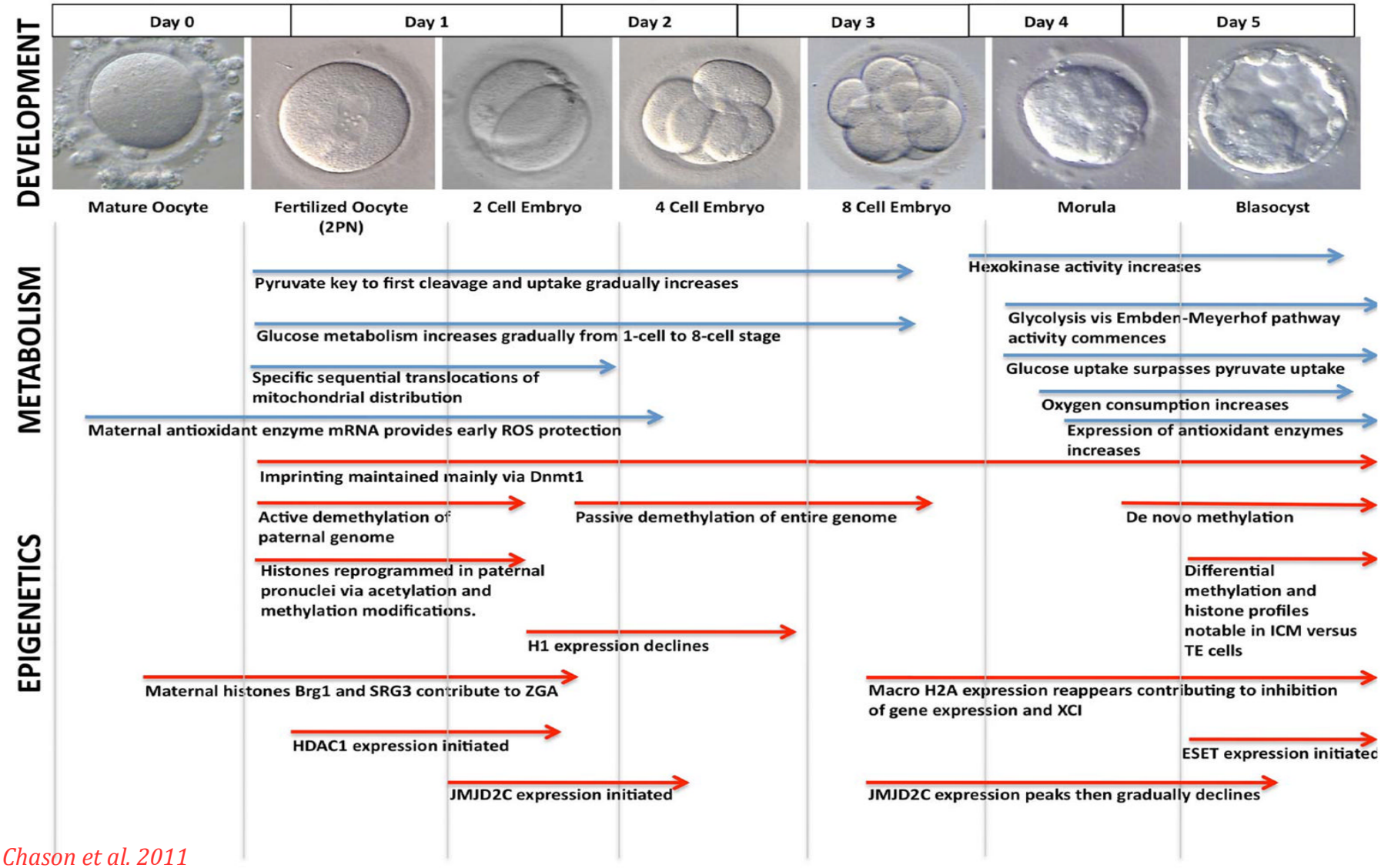
Interdependence of Metabolism and Epigenetics



R. J. Chason et al. 2011

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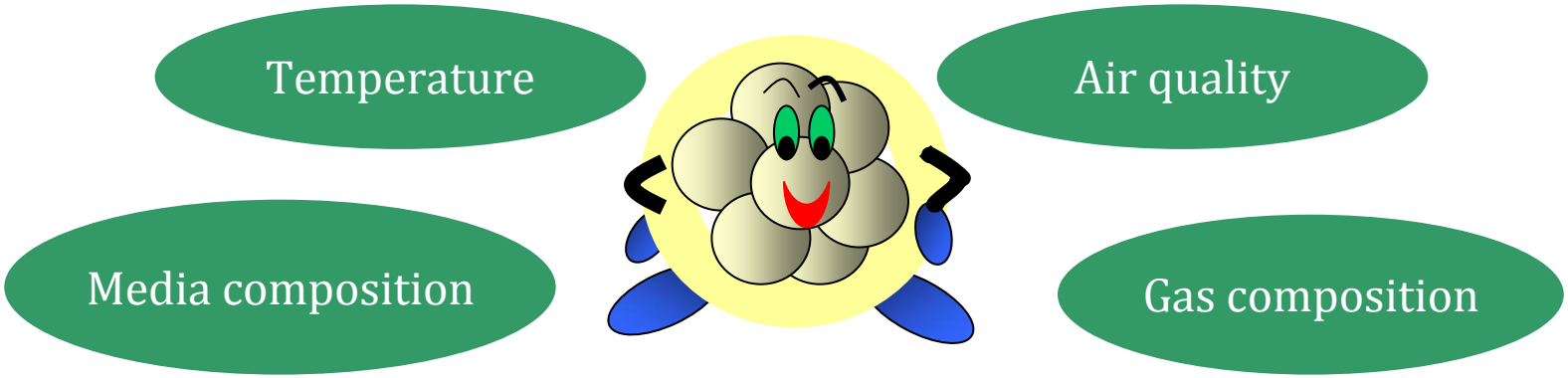
Introduction to culture system

Whatever culture system is chosen, a key to successful embryo culture is to minimize perturbations in the **micro-environment** around the embryo.



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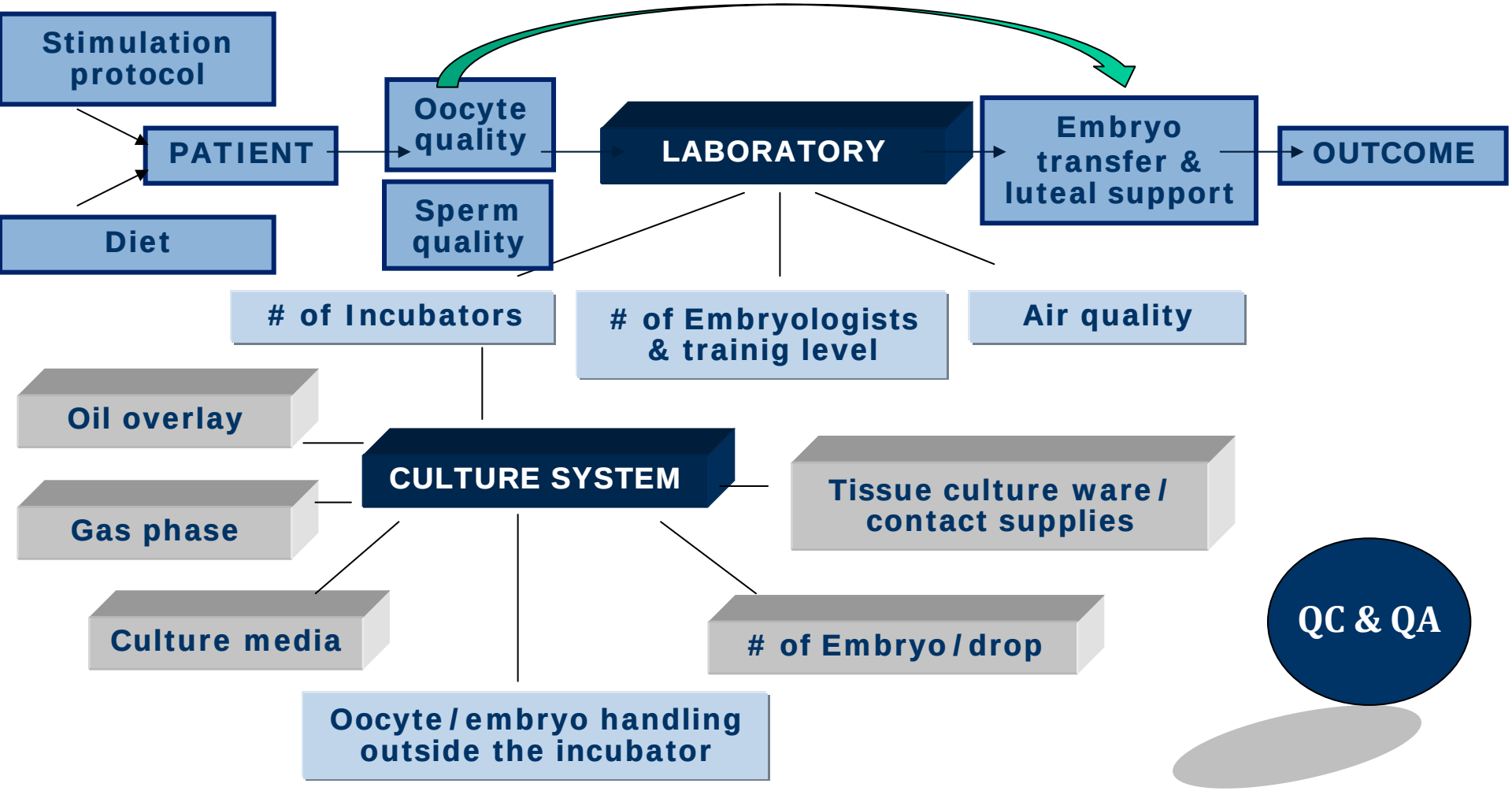
The central role of the laboratory



Gardner and Lane, 2007

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Biophysical factors

Identification of appropriate culture conditions focalizing on BIOPHYSICAL FACTORS

37° C

±0.2° C

pH

ROS production and REDOX state

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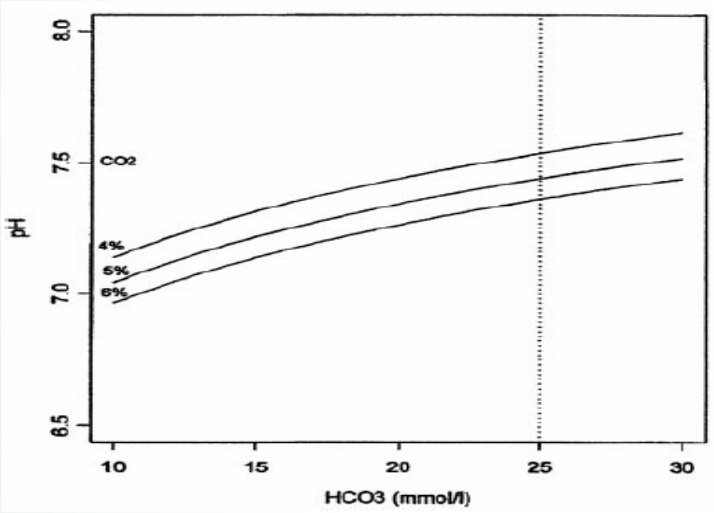
Buffer System and Hydrogen Ion Concentration (pH)

pHi in somatic cells is implicated in:

- metabolism
- activity of regulatory enzymes
- maintenance of gap junctions and the cyto-skeleton
- modulation of calcium levels
- proliferation

The pH and hence the [H+] can be approximately estimated using the Henderson-Hasselbach equation: pH = 6.1 + log [[NaHCO3]/(PaCO2)]

$$HCO_3^- + H^+ \leftrightharpoons H_2CO_3 \leftrightharpoons CO_2 + H_2O$$



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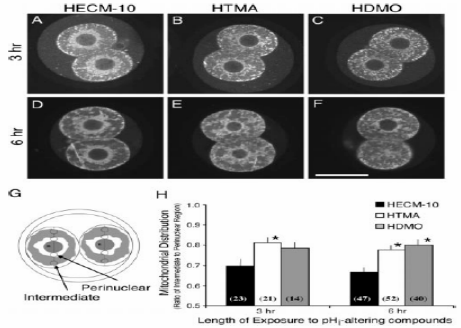
Effects of pH alteration

It has been established that even relatively small fluctuations in pHi can significantly retard subsequent developmental competence

Compound concentration[a]	pH_i[b]	% Morula and blastocyst	% Blastocyst
TMA 0 mM	7.186 ± 0.04	97.3	73.3
TMA 5 mM	7.285 ± 0.07	82.6[c]	34.7[d]
TMA 10 mM	7.383 ± 0.04[c]	49.1[d]	6.0[d]
TMA 20 mM	7.421 ± 0.06[c]	5.17[d]	2.6[d]
TMA 40 mM	7.538 ± 0.04[d]	0[d]	0[d]
TMA 80 mM	7.685 ± 0.05[d]	0[d]	0[d]
DMO 0 mM	7.242 ± 0.04	90.58	74.7
DMO 5 mM	7.221 ± 0.07	87.1	61.1[c]
DMO 10 mM	7.09 ± 0.05[c]	46.3[d]	21.6[d]
DMO 20 mM	6.87 ± 0.05[d]	0[d]	0[d]

Altering pHi disrupts the organization of mitochondria



Bavister et al 2001, Edwards et al,1998, Squirrell et al. 2001

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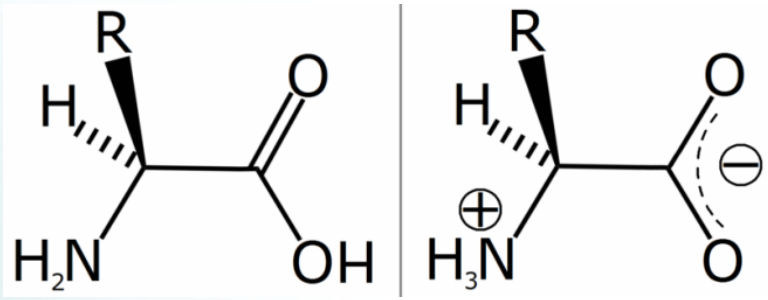
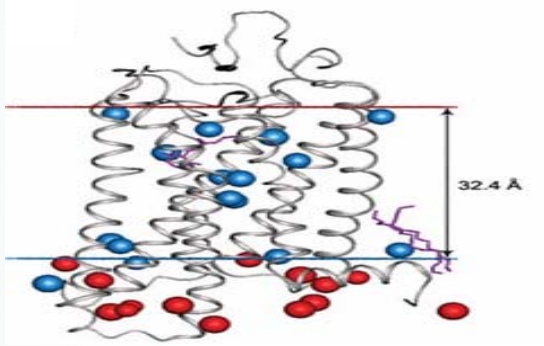
Cellular buffer systems

- At cytoplasmatic level, locally and rapidly by intrinsic buffers such as the zwitterionic amino acids



- Ionic membrane transport mechanisms



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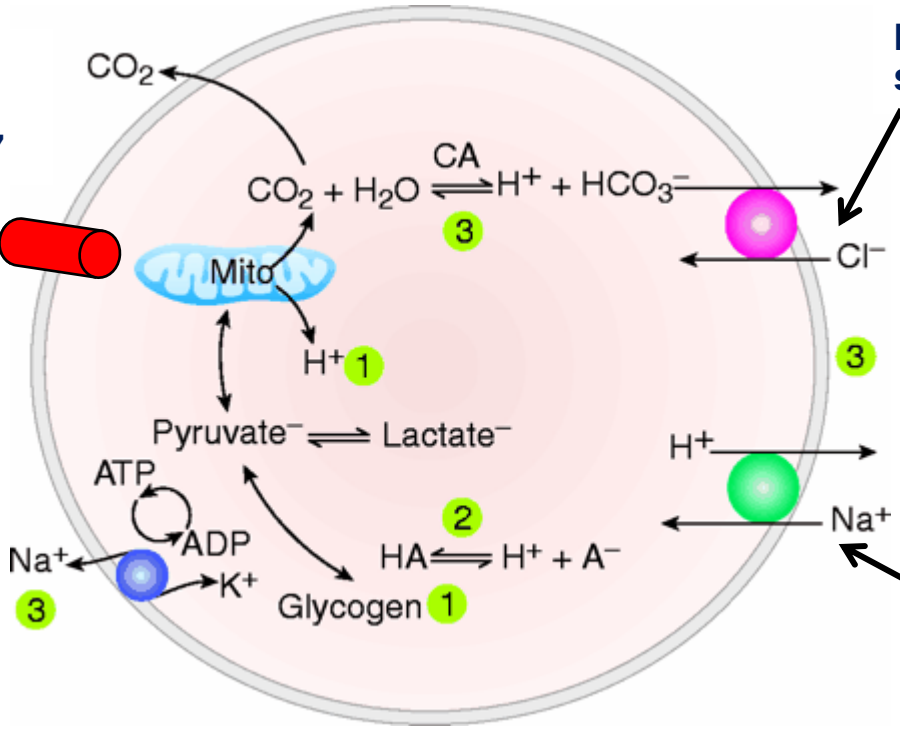
Cellular buffer systems

Three transporters collectively maintain the pHi of blastomeres at a set point that falls between 7.0 and 7.3, provided that HCO3 and CO2 are present, not as conjugates of the buffer system, but as components of the cellular ion exchangers.

Na+ / Cl-HCO3-
Relieves acidosis with set point pH<7

25mM
Cl^-
Na^+
$HCO3^-$
CO2



Relieves alkalosis, with set point 7.3 pHi

Relieves acidosis, with set-point 6.8 pHi

~~HEPES~~
~~5mM ions~~

Phillips et al. 2000

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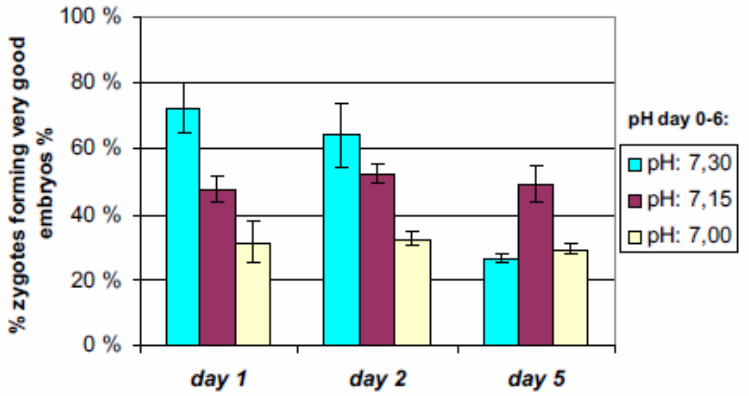
Differential pH in embryo culture

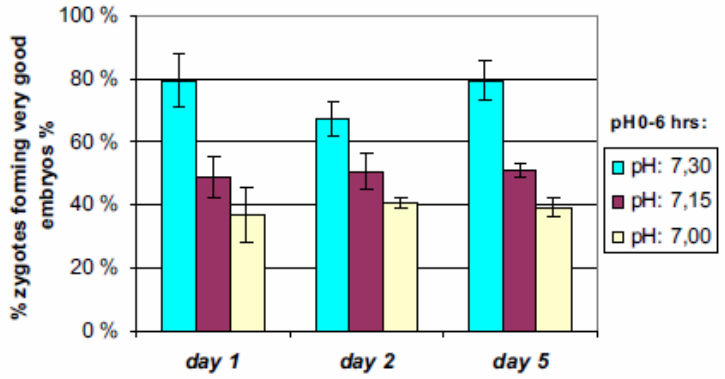
A **high pH during the first 6 hours and a lower pH during embryo culture** resulted in a significantly **improved blastocyst development**

Three sets of embryo cultures with three pH values over the whole culture period. The media were changed on day 2. The three different colors represent three culture sets.



Hentemann. Differential pH in embryo culture. Fertil Steril 2011.

Three sets of embryo cultures and three different pH values during the first 6 hours. Media were changed at 6 hours to obtain pH 7.15 in all cultures for the remaining culture period. Three colors represent three culture sets.



Hentemann. Differential pH in embryo culture. Fertil Steril 2011.

pH 7.30 was found to be the optimum up **to the pronuclear stage**

pH 7.15 for **cleaving embryos**

Hentemann et al., 2011

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Biophysical factors

Identification of appropriate culture conditions focalizing on BIOPHYSICAL FACTORS

O2

ROS production and REDOX state

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OXIDATIVE STRESS

Potential sources of oxidative stress:

- Exposure to light
- Presence of transitional metals in the culture media
- High oxygen tension (i.e., 20%)

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Culture system: Gas Phase

Human *in vitro* embryo culture is reported to be performed using a gas phase containing either atmospheric (**~20%**) or reduced (**~5%**) **oxygen concentrations.**

High oxygen concentration has been shown to adversely affect **embryonic development** in several animal species. Quinn & Harlow 1978, Batt et al., 1991, Fujitani et al., 1997; Gardner et al., 1996; Karagenic et al., 2004; Booth et al., 2005

No beneficial (or too marginal) effect of low O2 concentrations has been reported in IVF programs. Dumoulin et al., 1995, 1999

More recent data have, however, underlined the importance of the Gas composition for human embryo culture.

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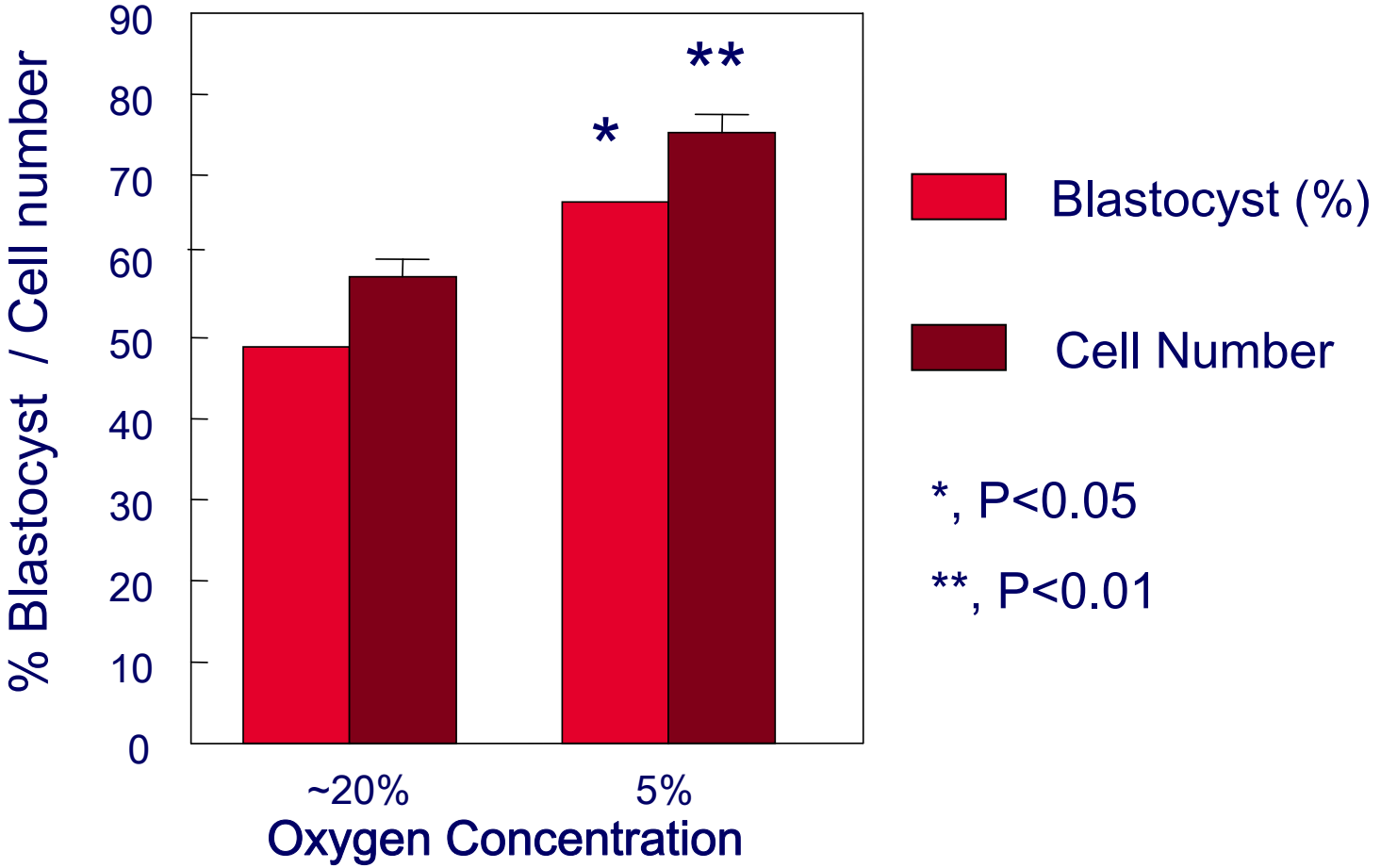
Effect of Oxygen concentration on mammalian embryonic development

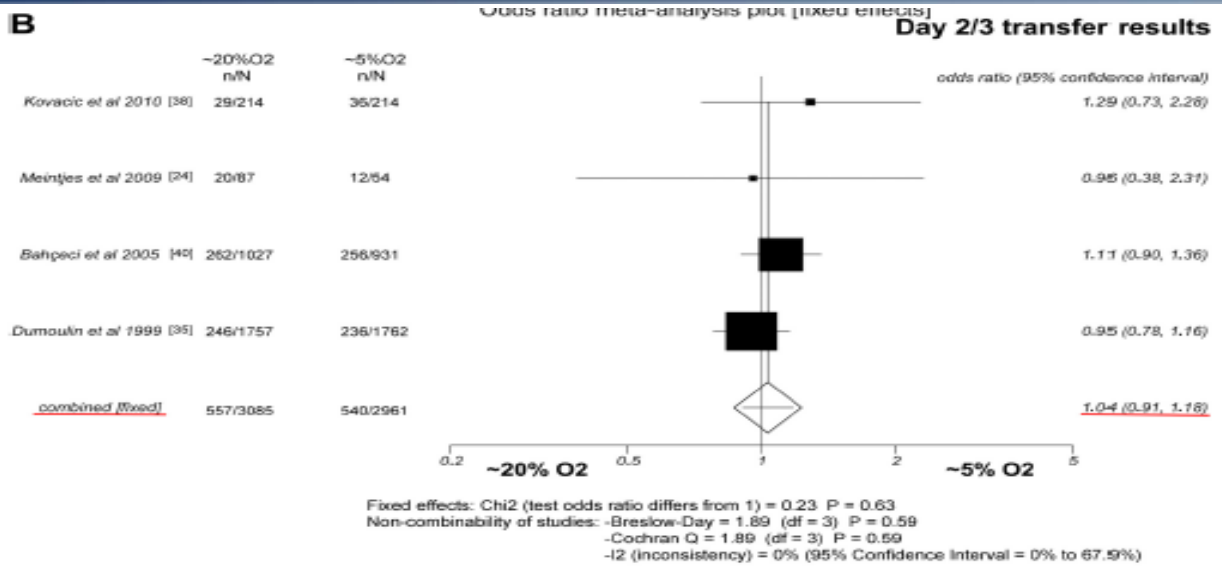


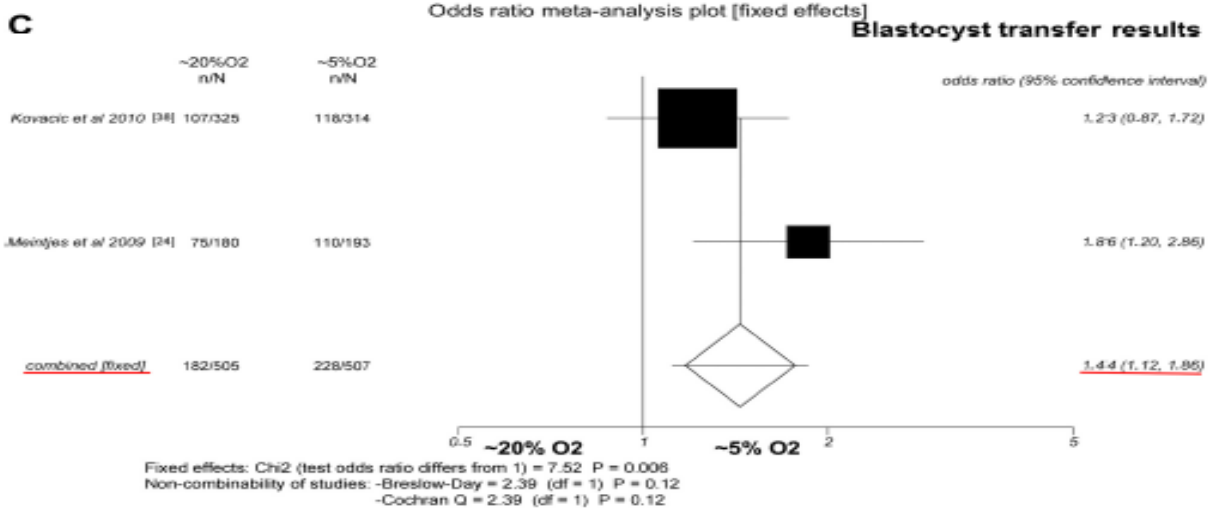
Gardner et al. personal comunication

REVIEW Open Access

IVF/ICSI outcomes after culture of human embryos at low oxygen tension: a meta-analysis

David B Gomes Sobrinho[1], Joao Batista A Oliveira[1,2,3*], Claudia G Petersen[1,2,3], Ana L Mauri[2,3], Liliane FI Silva[1,2,3], Fabiana C Massaro[2,3], Ricardo LR Baruffi[2,3], Mario Cavagna[2,3,4] and José G Franco Jr[1,2,3]





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Effect of Oxygen concentration on mammalian embryonic development

Even **transient exposure** to high O2 concentration reduce embryo development in vitro

Culture dishes **equilibrated** at 20% oxygen concentration for five hours prior culture in low O2 concentration decreases mouse zygote development to the blastocyst stage and resultant blastocyst cell number

IT TAKES MORE THAN 5 HOURS FOR THE OXYGEN CONCENTRATION TO FALL TO EMBRYO SAFE LEVELS!!

Gardner, personal comunication

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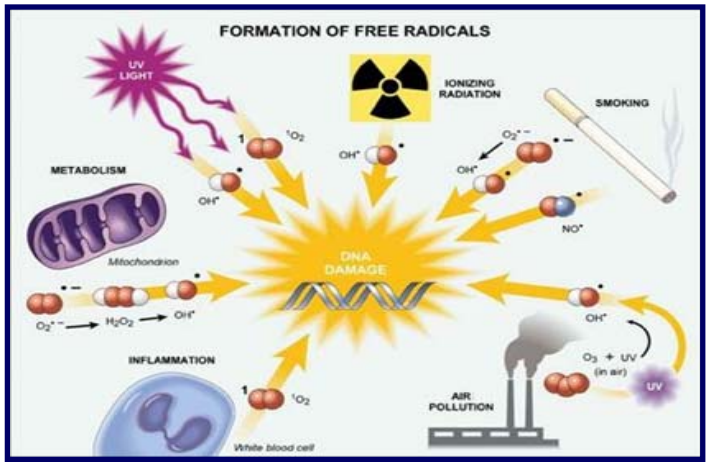
Oxygen concentration

Under atmospheric oxygen conditions the main contributor to poor embryo development is supposed to be ROS production. However a 'cause and effect' mechanism is yet to be demonstrated.



- **There are some specific events in reproductive system that are positively associated with ROS production**
- **Increase in antioxidant gene expression under 20% oxygen has not been observed**
- **Reducing O2 tension is more effective in promoting embryo development *in vitro* than is treatment with detoxifying enzymes (superoxide dismutase and catalase)**

Harvey et al., 2002; Thomas et al., 1997

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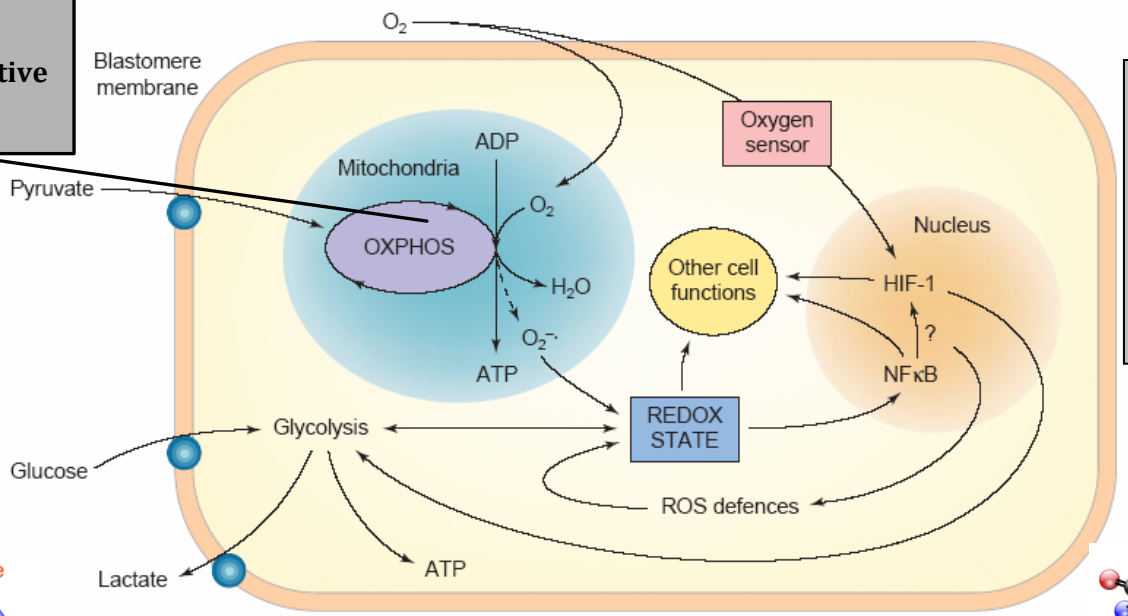
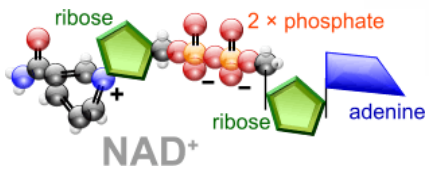
REDOX state

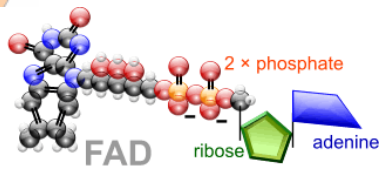
REDOX state is considered to be an important mechanism that regulates several cell functions, particularly in the preimplantation embryo.

Oxygen is an essential key energy substrate for oxidative phosphorylation

a key modulator of metabolic pathways (OXPHOS and glycolysis)







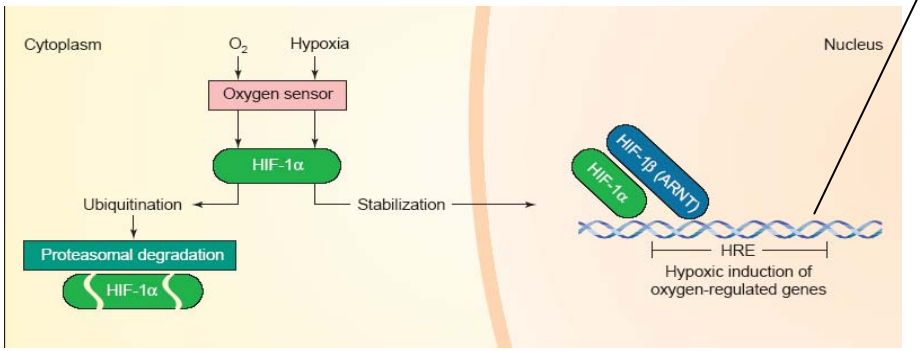
Other cellular functions, such as apoptosis and cell cycle control, are also significantly influenced by oxygen availability and REDOX state, via transcription factors such as NFkB

Harvey et al., 2004

Hypoxia-inducible factors

Members of the hypoxia inducible factor family of transcription factors are influenced directly by the intracellular oxygen concentration, and are important for embryonic development within the hypoxic reproductive tract

Under normoxic conditions, HIF- 1a protein is degraded rapidly by the ubiquitin–proteasome system



Core consensus sequence (A/G)CGTG in the hypoxia response element (HRE) regulatory region

HIF1 regulated genes (adapted from Semenza, 2002 and Bracken et al., 2003)

Gene product	Function
Adenylate kinase 3	Glucose uptake/glycolysis
α1B-adrenergic receptor	Angiogenesis
Adrenomedullin	Angiogenesis
Aldolase A, C	Glucose uptake/glycolysis
Carbonic anhydrase 9	Glucose uptake/glycolysis
Ceruloplasmin	Iron metabolism
Collagen type V, α1	Extracellular matrix
Cyclin G2	Proliferation and survival
Cyclooxygenase-2 (COX-2)	Various functions
DEC-1, 2	Proliferation and survival
Endothelin-1	Angiogenesis
Enolase 1	Glucose uptake/glycolysis
Erythropoietin	Iron metabolism
ETS-1	Transcription factor
Glucose transporter 1 (GLUT1), 3	Glucose uptake/glycolysis
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	Glucose uptake/glycolysis
Heme oxygenase 1	Angiogenesis
Hexokinase 1, 2	Glucose uptake/glycolysis
Insulin-like growth factor 2 (IGF2)	Proliferation and survival
IGF binding protein 1 (IGFBP1)	Proliferation and survival
IGFBP2, 3	Proliferation and survival
Inducible nitric oxide synthase 2 (iNOS)	Angiogenesis
Lactate dehydrogenase A (LDHA)	Glucose uptake/glycolysis
LDL receptor-related protein 1	Various functions
NIP3	Proliferation and survival
NIX	Proliferation and survival
p21	Proliferation and survival
6-Phosphofructo-2-kinase/fructose-2,6-biphosphatase-3	Glucose uptake/glycolysis
Phosphofructokinase L	Glucose uptake/glycolysis
Phosphoglycerate kinase 1	Glucose uptake/glycolysis
Plasminogen activator inhibitor 1	Angiogenesis
Prolyl-4-hydrogenase α(I)	Metabolism
Pyruvate kinase M	Glucose uptake/glycolysis
Transferrin, transferrin receptor	Iron metabolism
Transforming growth factor β_3	Proliferation and survival
Transglutaminase 2	Protein modification
Triosephosphate isomerase	ATP metabolism
Vascular endothelial growth factor (VEGF)	Angiogenesis
VEGF receptor flt-1	Angiogenesis

Under hypoxic conditions, the HIF-1a protein is not targeted for degradation and can translocate to the nucleus to form the active DNA-binding complex

$[O_2]$ → **Correct gene expression**

Semenza et al., 2000

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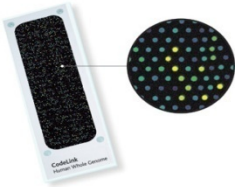
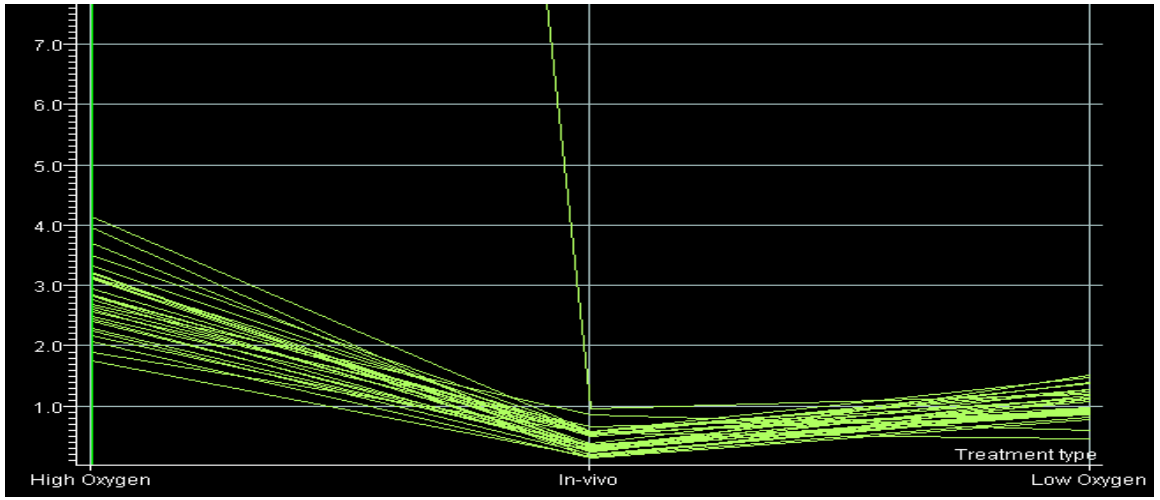
Effect of Oxygen concentration on gene and protein expression (mammalian embryos)

A recent **proteomic analysis** of mammalian preimplantation embryonic development revealed that specific pattern of 10 biomarkers effectively discriminated *in vitro* embryos cultured at low or high oxygen concentration





High O_2 In-vivo Low O_2

Katz-Jaffe et al., 2005

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What should we ensure for embryos?

IN THE INCUBATOR

- Low O2 concentration **must** be used for human embryo culture

- CO2 concentration depend on the culture media used and should be monitored in the incubator

- pH should be routinely controlled in the culture media

- Temperature fluctuations should be avoided (reduce door opening-**lab setting**)

- The air should be filtered and purified (or use of pre-mixed gas is advisable)

-Critical items of equipment should be appropriately alarmed and monitored

OUTSIDE THE INCUBATOR

- Reduce to minimum dishes exposure to external conditions

- during handling/evaluations the use of “chambers” are recommended

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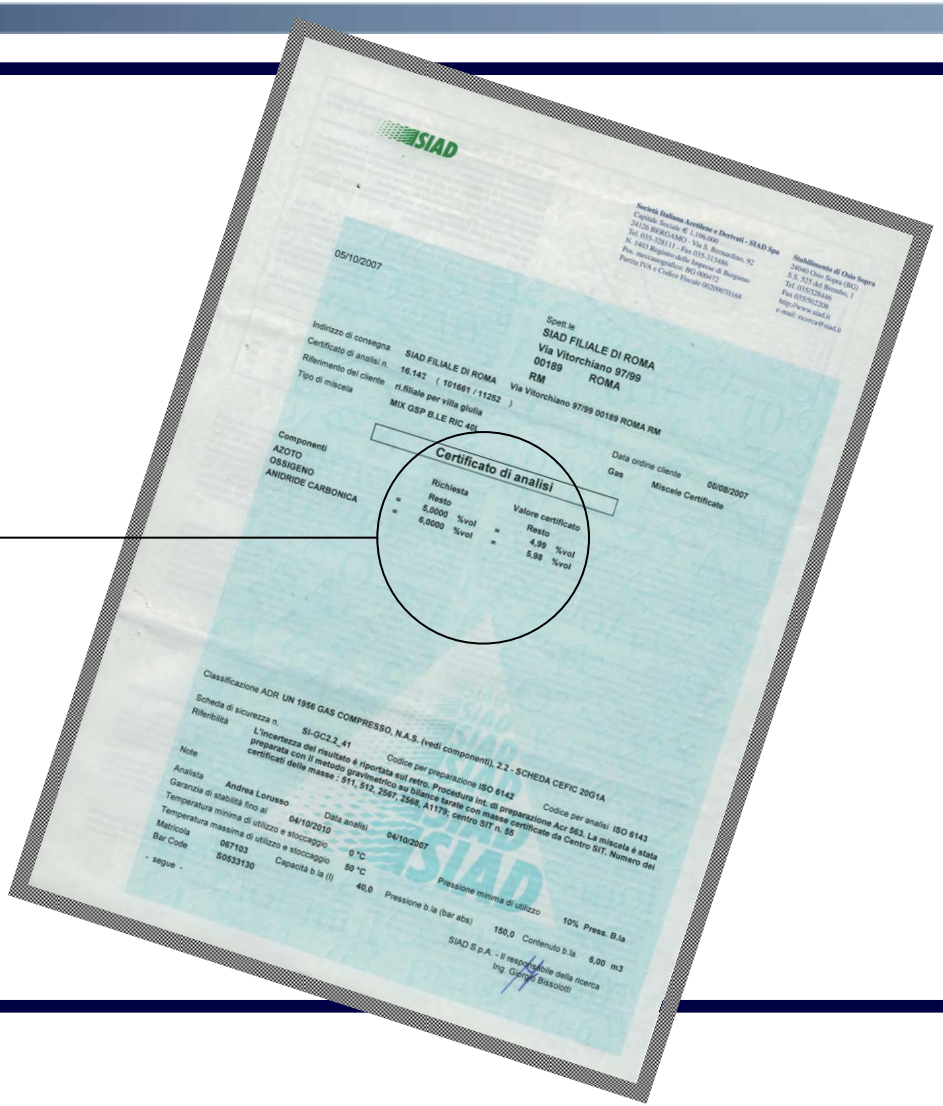
PRE-MIXED GAS: certification

99,99 PURETY

4.99 % O2

5.99 % CO2

Rest N2



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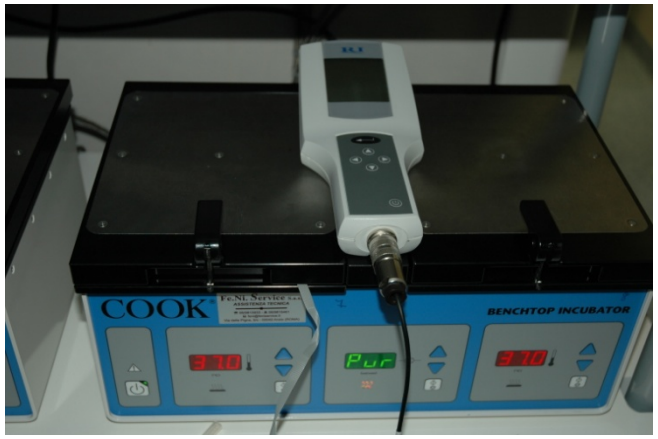
Monitoring with independent probes

•Regular (daily) checks of functional parameters for devices used to maintain temperature and CO2 should be performed using calibrated thermometers and extra methods of CO2 analysis and/or pH measurement.

Gas analyser

Thermometer



pH analyser

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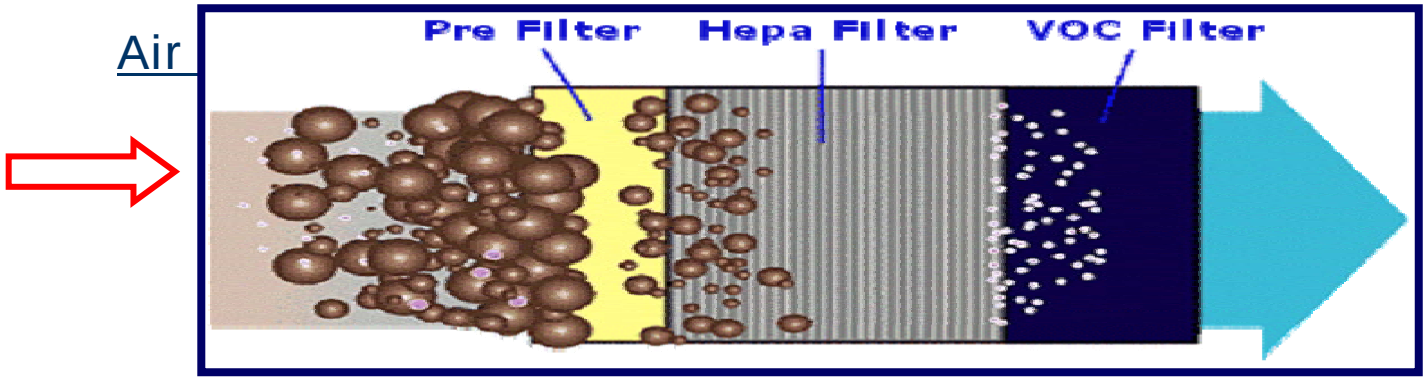
Air quality

The Air in the Lab. can contain: Bacteria & Viruses, Mold, Fungus, Cleaning Chemicals, Paint, Solvents, Ozone & Smog, Nitrous Oxide, Hair Spray, Perfume, Pesticides, Alcohols, Ammonia, Chlorinated Solvents, Carbon Monoxide



A large proportion of the air inside the incubators is ambient air from the laboratory. Therefore, maintaining good-quality ambient air in the laboratory also affects the quality of the air contained in incubators.

This is not especially relevant when using bench top incubators

TOWARDS NEW DEVICES?

Design of a workstations that provides a safer and

more standardised environment, in terms of gas composition and temperature stability, during handling and evaluations performed outside the controlled environment of the incubator.

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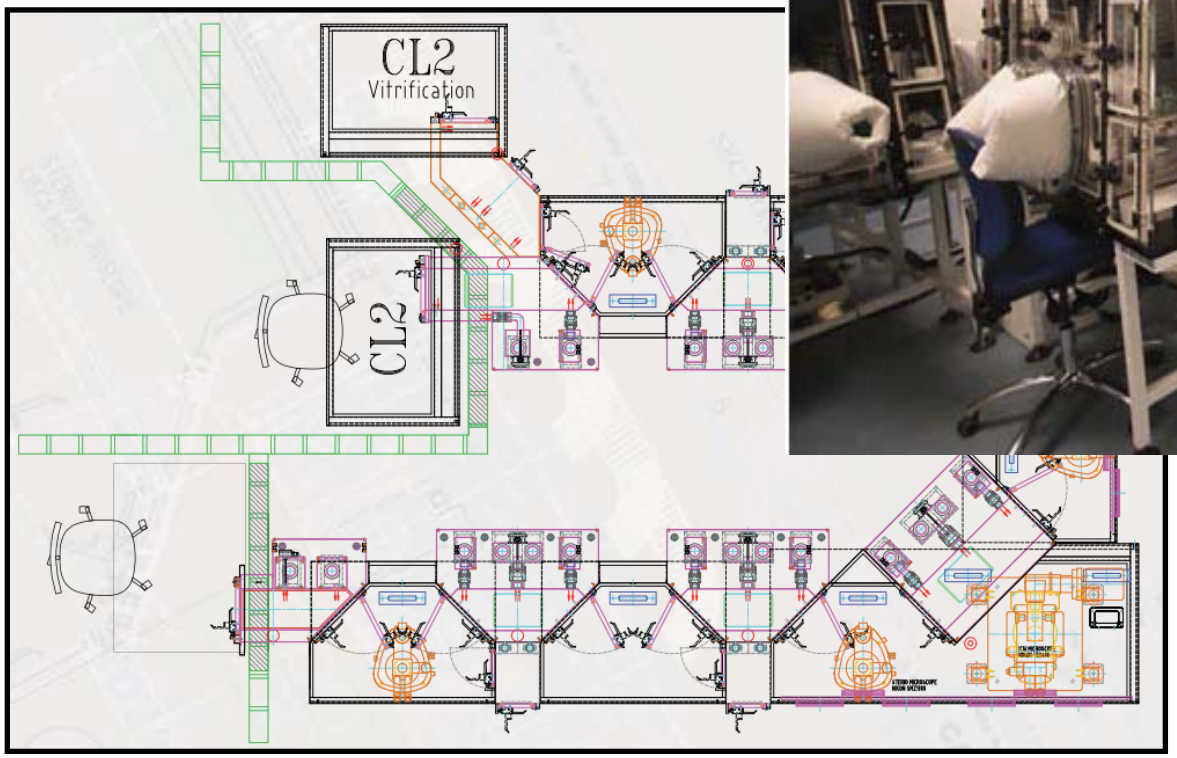
TOWARDS NEW DEVICES?

TOTALLY ENCLOSED ENVIRONMENT



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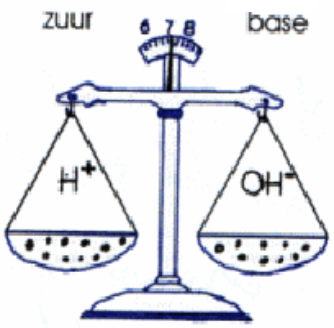
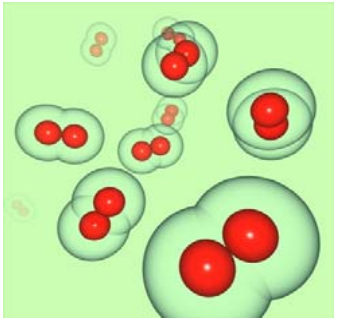
Take home message





Temperature should be stable around 37°C +0.2°C

Extracellular pH should range between 7.15-7.35

Slight variation in pHi induces:

1. Drastic decrease in blastocyst development
2. Embryo metabolism modification
3. Altered perinuclear mithocondrial distribution

Oxygen concentration should be physiologycal in the gas phase because it influences:

1. REDOX state of the cells
2. Gene expression
3. Embryo metabolism and differentiation

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Interdependence of Metabolism and Epigenetics

The "*quiet embryo hypothesis*" proposes that viable embryos are associated with a "quiet" rather than an "active" metabolism (Lane 2002)

This hypothesis could explain the link between embryonic metabolism, epigenetics and viability

If the epigenome serves as a link between the environment and the genome,
then ***in vitro*** **culture may impact embryo metabolism and development** by way of **alterations in the epigenetic marks** that regulate pertinent metabolic pathways