Institut für Tierwissenschaften, Abt. Tierzucht und Tierhaltung

der Rheinischen Friedrich-Wilhelms-Universität Bonn

Expression analysis of homeobox gene family in bovine pre- and post-implantative developmental stages

I n a u g u r a l-D i s s e r t a t i o n

Zur Erlangung des Grades

Doktor der Agrarwissenschaften

(Dr. agr.)

der

Hohen Landwirtschaftlichen Fakultät

der

Rheinischen Friedrich-Wilhelms-Universität

zu Bonn

vorgelegt im Mai 2006

von

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aus

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Landwirtschaftliche Fakultät - Jahrgang 2006
Zugl.: ITW; Bonn, Univ., Diss., 2006

Diese Arbeit wurde mit finanzieller Unterstützung vom CONACYT (Mexiko) durch der DAAD (Deutschland) umtausch Program zuzamen mit der Institut für Tierwissenschaften der Rheinischen Friedrich-Wilhelms-Universität Bonn durchgeführt.

D98

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Korreferent:	Prof. Dr. B. Petersen
Tag der mündlichen Prüfung:	30. Juni 2006

Expressionsanalyse der Homeobox-Genfamilie in bovinen prä- und postimplantativen Entwicklungsstadien

Homeobox- (Hox-) Gene kodieren Transkriptionsfaktoren, die die Expression verschiedener essentieller Proteine kodierender Zielgene für Zelldifferentation und -proliferation regulieren. Daher gelten Hox-Gene seit langem als Hauptregulatoren der Säugetierentwicklung. Das Ziel der vorliegenden Untersuchung war die Untersuchung der Expressionsprofile von Mitgliedern der Hox-Genfamilie in prä- und postimplantativen Entwicklungsstadien boviner Embryonen. Hierzu wurden ESTs von Cdx1, Cdx2, HoxA7, HoxB6, HoxB7, HoxB9, HoxC9, HoxD1 und HoxD4 sequenziert. Mittels quantitativer real-time PCR wurden die Expressionsprofile von Cdx1, Cdx2, HoxB7, HoxB9, HoxC9, HoxD1 und HoxD4 bestimmt. Analysiert wurden Triplikatpools (mit jeweils 10) immaturer Oozyten, maturierter Oozyten, 2-Zell-, 4-Zell-, 8-Zell-, 16-Zellstadien, Morula und Blastozysten. Darüber hinaus wurden die Transkripte in den Embryonen an Tag 21, 25, 32 und 39 bestimmt. Die höchste relative Abundanz konnte für Cdx1, Cdx2, HoxB7, HoxB9 und HoxC9 im Stadium der immaturen Oozyte beobachtet werden, die Expression war in den späteren Entwicklungsstufen downreguliert für alle Transkripte, ausgenommen HoxD1 und HoxD4, die zu einem höheren Level abundant in maturen Oozyten waren. Die Transkripte von Cdx2, HoxB7, HoxB9 und HoxC9 zeigten einen progressiven Anstieg nach dem 16-Zell- bis zum Blastozystenstadium. Es wurde beobachtet, dass das Transkript von Cdx2 einen abundanten Level nach der Implantation zeigt, was die potentielle Rolle in der Embryoimplantation belegen könnte. Die relative Cdx1 Abundanz zeigte einen progressiven Anstieg an Tag 21, 25, 32 und 39, ähnlich wie HoxB7. Die Expressionsprofile von HoxB9 und HoxC9 erscheinen gleichartig nach der Implantation, beide zeigten die geringste relative Abundanz an Tag 25 und die höchste an Tag 32, mit einem Abfall an Tag 39. HoxD1 und HoxD4 wiesen die geringste relative mRNA Abundanz an Tag 21 auf, mit einem Anstieg an Tag 32. An Tag 39 zeigte sich HoxD1 erniedrigt und HoxD4 erhöht. Zusammenfassend lässt sich sagen, dass Hox-Gene aktive Transkripte in bovinen prä- und postimplantativen Stadien sind, was ihre potentielle Involvierung in der frühen embryonalen Entwicklungen als nukleare Transkriptionsregulatoren stützt.

Expression analysis of homeobox gene family in bovine pre- and postimplantative developmental stages

Homeobox (hox) genes encode transcription factors which regulate the expression of several target genes encoding essential proteins for cell differentiation and proliferation. Thus, hox-genes have long been recognized as master regulators of the mammalian development. It was the objective of the current study to investigate the expression profile of hox-gene family members in pre- and postimplantation developmental stages of bovine embryos. For this, EST´s derived from Cdx1, Cdx2, HoxA7, HoxB6, HoxB7, HoxB9, HoxC9, HoxD1, and HoxD4 were sequenced. The quantitative real-time PCR was applied to determine the expression profiling of Cdx1, Cdx2, HoxB7, HoxB9, HoxC9, HoxD1, and HoxD4. Thus, triplicate pools (each containing 10) of immature oocytes, matured oocytes, 2-cell, 4-cell, 8-cell, 16-cell, morula and blastocysts were analysed. Moreover, transcripts were quantified in embryos of day 21, 25, 32, 39. The highest relative abundance was observed for Cdx1, Cdx2, HoxB7, HoxB9, and HoxC9 at the immature oocyte stage and expression was down regulated in the later developmental stages, except for HoxD1 and HoxD4 transcripts, which were abundant at higher level in mature oocytes. Transcripts of Cdx2, HoxB7, HoxB9, and HoxC9 were found to progressively increase after 16 cells stage until blastocyst stage. The Cdx2 transcript was found to have abundant level after implantation, which may support its potential role in embryo implantation. The relative abundance of Cdx1 was found to progressively increase at day 21, 25, 32, and 39, similarly as HoxB7. The expression profile of HoxB9 and HoxC9 genes seems to be similar after implantation, both showed the lowest relative abundance at day 25 and the highest at day 32, declining at day 39. HoxD1 and HoxD4 showed the lowest relative mRNA abundance at day 21, increasing at day 32. At day 39, HoxD1 appeared declined and HoxD4 was increased. In conclusion, the present study has shown that Hox-genes are active transcripts in bovine preimplantation and postimplantation stages, supporting their potential involvement in the early embryonic development as nuclear transcription regulators.

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List of abbreviations

A	:	Adenine
A_{260}	:	Absorbance at 260 nm
A_{280}	:	Absorbance at 280 nm
aa	:	Amino acid
ABI	:	Applied biosystems
AER	:	Apical ectodermal ridge
AI	:	Artificial insemination
Ala	:	Alanine
ANOVA	:	Analysis of variance
Antp	:	Antennapedia
AP	:	Antero-posterior
APS	:	Ammonium peroxide disulphate
Arg	:	Argynine
ART	:	Assisted reproductive technologies
BC	:	Body condition
BCM	:	Baylor college of medicine
BLAST	:	Basic local alignment search tool
BME	:	Basal medium Eagle
bp	:	Base pairs
BSA	:	Bovine serum albumin
C	:	Cytosine
$CaCl_2$	:	Calcium chloride
cAMP	:	Cyclic adenosine mono phosphate
CCD	:	Charge-coupled device
cDNA	:	Complementary DNA
CO_2	:	Carbonic dioxide
COCs	:	Cumulus oocyte complexes
CP	:	Crossing point
CR	:	Conception rate
CR1aa	:	Charles Rosenkrans 1 medium with amino acids
CREB	:	Amino-terminal domain of CBP binding protein
C_T	:	Threshold cycle
Cu/Zn-SOD	:	Cupper-zinc superoxide dismutase

d	:	Day
Da	:	Daltons
dATP	:	Desoxy thymin triphosphate
dCTP	:	Desoxy cytosine triphosphate
ddATP	:	Didesoxy thymin triphosphate
ddCTP	:	Didesoxy cytosine triphosphate
ddGTP	:	Didesoxy guanidine triphosphate
ddH_2O	:	Didestilled water
ddUTP	:	Didesoxy uracile triphosphate
DEPC	:	Diethyl pyrocarbonate
DF	:	Dilution factor
dGTP	:	Desoxyguanine triphosphate
DMSO	:	Dimethylsulfoxide
DNA	:	Deoxyribonucleic acid
DNase	:	Deoxyribonuclease
dNTPs	:	Desoxynucleotid triphosphate
D-PBS	:	Dulbecco`s phosphate buffered saline
dpf	:	Days post fertilization
dpi	:	Days post insemination or matting
dsRNA	:	Double-stranded RNA
DTT	:	1, 4, dithio theritol
dTTP	:	Didesoxy thymin triphosphate
E_2	:	Estrogens
EAA	:	Essential amino acids
EB	:	Energy balance
eCG	:	Equine chorionic gonadotrophin
EDTA	:	Ethylene diamino tetra acetic acid
EGF	:	Epidermal growth factor
EST	:	Expressed sequence tag
EtBr	:	Ethidium bromide
FA	:	Formaldehyde
FCS	:	Fetal calf serum
FGFs	:	Fibroblast growth factors
FSH	:	Follicle stimulating hormone

G	:	Guanine
g	:	Gram
gDNA	:	Genomic DNA
GLM	:	General linear model
GS	:	Gene specific
GSS	:	Gene species specific
H_2O	:	Water
hCG	:	Human chorionic gonadotrophin
HCl	:	Hydrochloric acid
HEPES	:	4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid
Hox	:	Homeobox
i.d.	:	Identity
hpi	:	Hour(s) post insemination
hr	:	Hour(s)
ICM	:	Inner cell mass
IMO	:	Immature oocyte
IPTG	:	Isopropyl ß-D-thiogalactoside
IU	:	International units
IVEC	:	In vitro embryo culture
IVF	:	In vitro fertilization
KCl	:	Potassium chloride
L	:	Litre
LB-Broth	:	Luria-Bertoni broth
LiCl	:	Lithium chloride
LOS	:	Large offspring syndrome
Lys	:	Lysine
mA	:	Milli-Ampere
MEM	:	Minimal essential medium
mg	:	Milligram
$MgCl_2$	:	Magnesium chloride
Min(s)	:	Minute(s)
ml	:	Millilitre
mm	:	Millimetre
mM	:	Millimolar

MO	:	Mature oocyte
MPM	:	Modified parker medium
mRNA	:	Messenger RNA
Mw	:	Molecular weight
N_A	:	Avogadro constant
NaCl	:	Sodium chloride
$NaHCO_3$	:	Sodium carbonate
NCBI	:	National center for bioinformation
NEA	:	Nonessential amino acids
ng	:	Nanogram
nM	:	Nanomolar
nmol	:	Nanomole
nt	:	Nucleotide
NTC	:	Non template control
O_2	:	Oxygen
OCS	:	Oestrus cow serum
OD	:	Optical density
OPU	:	Ovum pick up
P_4	:	Progesterone
pDNA	:	Double-stranded plasmid DNA
PGC	:	Primordial germ cells
PGF	:	Peptide like growth factors
$PGF_2\alpha$	:	Prostaglandin F2α
PGs	:	Prostaglandins
PHE	:	Penicillamin-hypotaurin-epinephrine
Phyre	:	Protein homology/analog Y recognition engine
pmol	:	Picomole
Q	:	Quantity
RA	:	Retinoic acid
RAREs	:	Retinoic acid responsive elements
RARs	:	Retinoic acid receptors
recDNA-GS	:	Recombinant DNA gene specific
RCF/G	:	Relative centrifugal force / earth´s gravitational field
RNA	:	Ribonucleic acid

RNase	:	Ribonuclease
RNasin	:	Ribonuclease inhibitor
ROS	:	Reactive oxygen species
rpm	:	Rotations per minute
RT-PCR	:	Reverse transcriptase PCR
SDS	:	Sodium dodecyl sulphate
sec	:	Seconds
Ser	:	Serine
T	:	Thymine
TAE	:	Tris acetic acid EDTA
TALP	:	Tyrode's albumin lactate pyruvate
Taq	:	Termophilus acuaticus
TBE	:	Tris boric acid
TCM	:	Tissue culture medium
TD-PCR	:	Touch down PCR
TE	:	Trophectoderm
Tm	:	Melting temperature
Tris	:	2-amino-2(hydroxymethyl)propane-1,3-diol
TS	:	Trophoblast stem
TVFA	:	Transvaginal follicular aspiration
U	:	Units
UTRs	:	Untranslated regions
UV	:	Ultraviolet
v	:	Volume
V	:	Volt
v/v	:	Volume per volume
W	:	Watt
w/v	:	Weight per volume
X-gal	:	5-bromo-4-chloro-3-indolyl-ß-D-galactoside
X TE	:	Tris EDTA
µg	:	Microgram
µl	:	Microlitre
µM	:	Micromolar
µmol	:	Micromole

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1. Introduction

Oocytes maturation, gamete survival, as well as fertilization are driven by molecular mechanisms establishing the early embryonic development, but until now a lot of such molecular pathways and related phenomena remain to be well studied (Boerjan et al. 2000, McEvoy et al. 2006, Sinclair et al. 2003a, b).

Bovine preimplantation embryogenesis is characterized for such morphological transitions and molecular pathways after fertilization, comprising the first cleavage division, when the potential development of the embryo could be indicated (Lonergan et al. 2003a, b). These morphological and metabolic transitions in the preimplantation development are regulated by differential expression of developmentally important genes (Schultz et al. 1999, Zimmermann and Schultz 1994). First cleavage divisions are controlled by maternal mRNA transcripts kept during oogenesis. In bovine, after 8 cells stage begins the activation of the proper embryonic genome (EGA) (Memili et al. 1998, Memili and First 1999). Later, the compaction of the morula and the formation of the blastocyst are controlled by a proper mRNA transcription (Hardy and Spanos 2000).

The expression of transcription factor genes plays an important role in oocyte maturation, pre- and postimplantation development (Mohan et al. 2004). Wrenzycki et al. (2005b) postulated that in the preimplantation of the embryo of bovine perhaps 15 700 genes are transcribed, similarly as in the murine preimplantation period. Homeobox proteins are transcription factors of key genes involved in cell differentiation and proliferation (Gehring et al. 1994, Johnson et al. 2003). This group of nuclear proteins works at the transcriptional level (Duprey et al. 1988), being essential in early preimplantation stages and post implantation development and along the whole life (Adjaye and Monk 2000). Some Hox-gene family members participate in the TE differentiation and later in the cell fate (Chawengsaksophak et al. 1997, Imakawa et al. 2004, Ralston and Rossant 2005, Strumpf et al. 2005, Tamai et al. 1999).

Characterizing the expression pattern of such transcription factors becomes necessary to win a lot of elements that could help to the study and establishment of molecular pathways, the understanding of the early embryonic development, and to improve assisted reproductive technologies (ART) (Thompson et al. 2002).

On the other hand, the expression patterns of many genes or proteins have been well established according to a range of international works (Laurincik et al. 2003, Lonergan

et al. 2004, 2006, Farin et al. 2001, 2004, 2006, Wrenzycki et al. 2005b). The quantification of the mRNA expression by means of RT-PCR technologies has been successfully applied in embryology. It helps to establish differences among stages of development and among environmental effects. As a result, step by step natural molecular interactions and implicated phenomena in the mammalian embryogenesis are being determined (Wrenzycki et al. 2005b).

For this, the identification of Hox-gene transcription factors is of relevant importance, because their involvement in the expression of target genes related with developmental pathways. So the quantitative expression profiling of Hox-gene members could be employed as a significant fact for the understanding of these effects observed in the development at early pre-, peri- and postimplantation stages. Hence, our objective was intended to do a molecular examination of these members of the homeobox gene family, which are differentially expressed during the early embryonic development, by means of RT-PCR technologies and the quantitative real-time PCR system. Hox-gene members could be employed as markers in order to present more information to the natural or abnormal developmental phenomena caused by the pressure of stressful embryonic surroundings.

2 Literature review

2.1 The role of homeobox genes in the mammalian development

2.1.1 Homeobox proteins and mammalian evolution

Lewis (1978) suggested that a group of genes, which he named “the bithorax gene complex” of Drosophila, is derived for duplication from an ancestral common gene. Such family was later denominated as the homeotic genes. Because mutations and changes among their members could cause modifications of complete segments or structures in the body developing in another one. Such effect is recognized as homeosis or homeotic transformation (Grier et al. 2005).

After that, McGinnis et al. (1984) revealed a surprising found about the relationship between the genome of insects and animals by way of the Homeobox (Hox) gene family. From such discovery many questions have been answered with respect to evolution. These since Hart et al. (1985) presented a direct evidence for the genetic expression of homeobox genes during early embryonic development, confirming the hypothesis of Lewis (1978) and explaining that Hox genes play an important role during embryogenesis by the control of the shape of the cell patterns impacting morphogenesis. Besides they observed a high conserved organization by the homeotic genes of drosophila in comparison to their equivalent human locus. Such genes have been conserved in a variety of invertebrates and vertebrates having an organization complexity and transcript expression similar as by the flies (Amores et al. 1998).

Soon afterwards, Breier et al. (1986) isolated and characterized a previously unknown member of the murine homeobox family with more homology to sequences obtained from Drosophila homeotic genes (the Antp) than any other known murine homeobox. One year latter Falzon et al. (1987) have achieved six homeobox containing DNA sequences from rats genome sharing more than 80% of homology at the nucleotide level, and more than 90% of homology at the amino acid level, in comparison with the homeobox from the Antp genes and from homeoboxes of other metazoans species.

The same research group established that some nucleotide sequences outside the homeodomain shared no homology with the Antp flanking regions, but others shared an equally high degree of amino acid homology within the homeobox and its immediate

flanking region with a putative homology by mouse genes. In the same work, Falzon et al. (1987) have shown by using northern blot analysis of rat RNA, that homeobox genes are expressed in a tissue-specific manner suggesting that some of the mammalian homeobox containing genes conserved along the evolution play important cellular and developmental functions (Adjaye et al. 1997, Adjaye and Monk 2000).

2.1.2 The homeodomain (homeobox)

Conventionally have been named Homeobox or Hox-genes, all genes which share in common the homeobox, being a DNA sequence motif encoding the homeodomain, a DNA-binding motif (Pendleton et al. 1993, Popodi and Raff 2001). They are 39 genes clustered in separated complexes on four separate chromosomes or linkage groups (A, B, C and D or 1, 2, 3, 4 or 5, etc.) and are grouped into 13 clusters positioned along the chromosome in the same order that they are temporarily activated and expressed. In general homeobox genes seem to have the same transcriptional orientation according to their sequence (3' to 5'), similarity and order along the chromosome (Chen and Capecchi 1999).

Even their spatial distribution of expression is extremely consistent among different species of metazoas (Burke et al. 1995, LaRonde-LeBlanc and Wolberger 2003). Still among the members of the same paralogous family, they have shown parallel and common characteristics of expression domains during the mammalian embryo patterning. So often present similar expression patterns and functions (Chen and Capecchi 1999, Houle et al. 2003, Manzanares and Krumlauf 2001, Pendleton et al. 1993, Taylor et al. 1997).

Hox-gene-transcript sequences have high homology in this known highly conserved region, which has been used to decipher the Hox gene family of different species as was made from the Antennapedia-class to human homeodomain. On this form, the early developmental characterization of this family of genes has been done in flies, frogs, chickens, fishes, rodents and humans (Murtha et al. 1991, Pendleton et al. 1993). Also the homeodomain has been the base for the classification of such proteins and their grouping in classes or paralog groups according to sequence similarities and chromosomal clustering (Gehring et al. 1994). In the NCBI data bank until now have been reported 226 human genes containing the homeobox motif.

2.1.3 Activities of the homeobox gene family

Homeobox genes are expressed in nuclear proteins which work at a transcriptional level by the three-dimensional structure of the homeodomain (Duprey et al. 1988). With respect to the activities of this family, the homeodomain has the form of a helix-turn-helix capable to bind at the promoter region of target genes in sequences like the 3´TAAT5´ (ATTA) core motif, known as TATA box (see figure 2.1) (Gehring 1987, Gehring et al. 1994, Johnson et al. 2003). Therefore homeotic proteins work as transcriptional factors of key genes involved in differentiation and proliferation of cells of many developmental process giving the cell fate in different tissues (Adjaye and Monk 2000, Gehring et al. 1994, Johnson et al. 2003).

Individually, Hox genes have multiple promoters that give rise to different transcripts expressed in diverse tissues (Aubin et al. 1998). So they are classically characterized as transcriptional activators and/or repressors for appropriate development of specific tissues, determining centrally a spatial regulation in the morphogenesis, growth and identity patterns of the body.

In mammals, the morphological regionalization arrangements in the early embryonic development, where the expression of hox-genes participates in a vastly integrated pathway, these together with neighbouring genes in the same linkage group, other paralogous genes, and even non paralogous genes, which are localized in separated clusters. Here all work together positively or negatively, but always in an analogous form with each other and not as an individual component. So it is forming a system to work collectively for a proper embryo regionalization along the body axes (Chen and Capecchi 1999). But their activity must be closely synchronized in a temporal-, spatial-, and gene specific manner because their global Hox-code function (Bondos et al. 2004).

Deschamps and van Nes (2005) have noted that the surrounding area of the hox-clusters directs the activity of the Hox-gene expression in a cell-tissue specific pattern, which could be independent in the described spatiotemporal Hox gene expression. They consider that the regulation of the Hox-genes at the early epiblast is independent of the Hox gene participation later in the development, because the Hox-gene expression derives from specific cells later differentiated.

Classically it has been established that homeobox containing proteins with the cooperation of Pbx proteins activate DNA by their binding in a specific sequence section, as previously mentioned (figure 2.1) (Gehring et al. 1994).

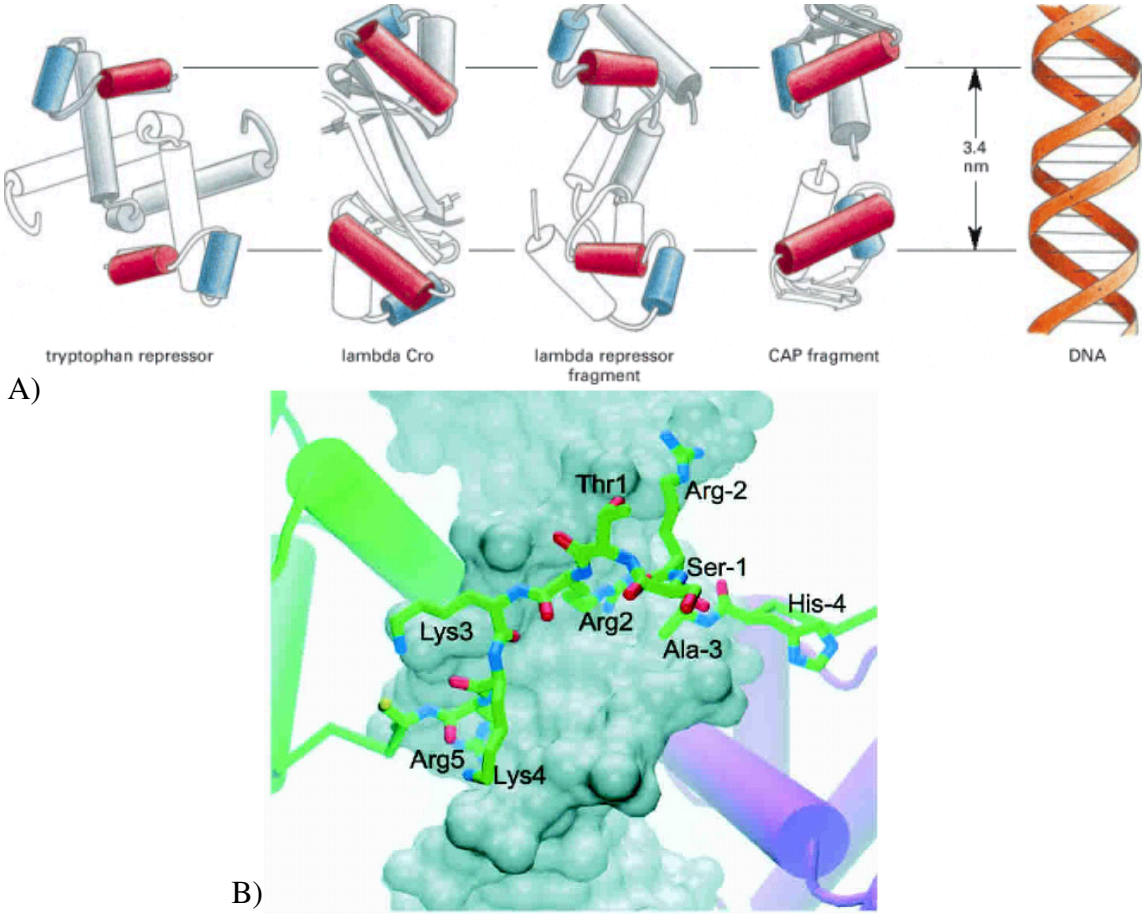


Figure 2.1 A) Example of some helix-turn-helix DNA-binding proteins of *E. coli* genes. These proteins bind DNA has dimers in which the two copies of the recognition helix (red cylinder) are separated by exactly one turn of the DNA helix, the external helix is coloured in blue. The helix-turn-helix motif controls the expression of many genes (Alberts et al. 2002).
B) Zoomed view of the linker region of the HoxA9 homeodomain N-terminal arm. The helix-3 residue of HoxA9 contacts the DNA major groove through the HoxA9 site 5´-$T_5T_6T_7A_8C_9G_{10}A_{11}C_{12}$-3´ across a minor groove making water-mediated contacts with Arg 2. The linker region is composed of Ala −3, Arg −2, and Ser −1, and Lys 4 where Ala −3 make contact to DNA sugar-phosphate backbones (LaRonde-LeBlanc and Wolberger 2003).

This binding is done through an YPWM motif and a protein complex. The motif is located in the N-terminal of the homeodomain, also found in the Cdx related members (IYPWMK) and other classes (Gehring et al. 1994).
But Bondos et al. (2004) in their work with flies have actually contributed with the discussion about the protein-protein interaction of the Hox family, supposing that to understand the functional discrimination classically observed by the Hox gene family, their protein interaction in variable regions could be an important key. Because their function in vivo needs an efficient specificity, that is provided by such interaction with heterologous proteins (Gehring et al. 1994, Sprules et al. 2003).
Hox-proteins can also bind a C-terminal dsRNA domain interacting with RNA. The protein binding is through a fold of the RNA-binding domain of a ribosomal protein structurally analogous to the homeodomain protein containing RNA recognition motifs. This has been observed by several hox-proteins binding RNAs for the anterior-posterior patterning by Drosophila embryos (Bondos et al. 2004).

2.1.4 Hox genes in early development

The main characteristic of the Hox-gene expression is their participation in the control of a lot of essential structural and functional activities in the development. These have been demonstrated in early epiblast and the trophectoderm (TE) differentiation, in the hindbrain establishment, in the nervous central system, as well as in cord and limbs, also by axial skeleton and organs like the gut, lungs and hematopoietic system, and along of the anterior-posterior axis of the embryo.
The Hox-gene expression is observed in a well structured colinear and sequential order. Their expression has to be spatially and temporally restricted for a proper patterning of vertebrate embryos, and still all along of the life (Beck et al. 1995, 1999, 2000, Burke et al. 1995, Charité et al. 1998, Chawengsaksophak et al. 1996, 2004, Deschamps et al. 1999, Isaacs et al. 1998, Kappen 2000).
The expression of most of the Hox-gene members appears earlier during embryogenesis, from very early stages in mammalian development (Ralston and Rossant 2005, Strumpf et al. 2005, Tamai et al. 1999). They have important roles in the cell differentiation, as presented at all the three embryonic germ layers as ectoderm, mesoderm and endoderm, as well after gastrulation. Also Hox-gene members would be

expressed in a cell specific form in adult cells requiring a constant transformation (Deschamps and van Nes 2005, Meyer and Gruss 1993).

Thus Murtha et al. (1991), Verlinsky et al. (1995), and Adjaye et al. (1997, 1999) reported the expression of these orthologous hox-genes expressed in plants, invertebrates and vertebrates, playing a crucial role in oocytes and the very early embryonic development. In such publications were detected and analysed homeobox containing genes among other member of the family in cDNA libraries generated from mouse and human oocyte and periimplantation stage embryos, as well as in old whole fetus. Later Adjaye and Monk (2000) detected for the first time HOXD1, HOXD8, HEX and OCT1 gene expression in the human oocyte and early stages of human development.

On a similar way, Ponsuksili et al. (2001a) reported for the first time the presence of the homeobox gene family in bovine tissue. In the same year, Ponsuksili et al. (2001b), have published that bovine Homeobox-genes could be expressed in bovine oocyte and preimplantative bovine embryo cultured in vitro in a similar pattern of expression as previously reported by mouse and human.

In the works of Ponsuksili et al. (2001a, b) was detected the transcription of Hoxd1 in bovine oocyte, interestingly together with Hoxa3. As well it was found the expression of Cdx1 and Cdx2 in two cells bovine embryos. Cdx1, Hoxa1, Hoxd1, and Hoxd4 were detected in four cells stage. From eight cells stage were amplified Cdx1, Hoxa1, and Hoxc9. In morula stages embryo has been found the expression of Cdx2, Hoxb9, and Hoxc9. Also they detected Cdx2, Hoxb7, Hoxb9 and Hoxc9 in blastocyst stages embryo. Whereas Verlinsky et al. (1998) reported mRNA expression of OCT-3, but not of HOXA4 in recollected post IVF blastocyst. These results consider with the previously detected by them self in 1995 by preimplantative human embryos, which did not express HOXA4 and HOXA7.

Thus in the research of Strumpf et al. (2005) it is reported that for the correct cell fate specification and differentiation of TE and inner cell mass (ICM) in the early blastocyst, homeobox containing genes are essential. So Cdx2 seems to be pivotal for implantation, taking the control of the lineage-restricted expression of Oct4 and Nanog at such stage (Chawengsaksophak et al. 1997, Tamai et al. 1999, Ralston and Rossant 2005).

The homeobox members, mouse and human Nanog, can be detected in the ICM of the blastocyst, after implantation in the proximal epiblast region of the presumptive

primitive streak, extended gastrulation and remaining restricted to epiblast, in embryonic gonads and in several adult tissues (Hart et al. 2004). Other homeobox members, as Oct members, are expressed in the epiblast and mesoderm at gastrulation, their expression could be observed more closely to Mixl1 or Otx2. Thus all they are implicated with other homeobox containing genes in early embryonic patterning and body fate determination.

For example, with the work of Pitera et al. (2001) it as been described the participation of the HOXD genes, formerly known as HOX5 and later as HOX4 in Drosophila, throughout the oogenesis and embryogenesis in mice, involving maternal and embryonic transcription. Later in the embryogenesis, Hoxd members participate in the hindbrain patterning, as well as in the endoderm and mesoderm adjacent to the caecum, also in developing ovaries, testicles, kidney and the gut.

This group, as typically in the family, has revealed an important role in the development of the appendicular skeleton system. In other words, they are related to differentiation and control of the patterning of the body plan in vertebrates, implicated in limb formation in most of the analysed species (Chawengsaksophak et al. 1996, 1997, 2004, Deschamps et al. 1999, Isaacs et al. 1998, Manzanares et al. 2000). Its expression has been well identified in a variety of embryonic connective tissues, including tendons and ligaments of limb buds (Burke et al. 1995, Khoa et al. 1999, Dealy and Kosher 1996, Zakany et al. 1997).

In mice and chicks Hoxa9, Hoxb9 and Hoxc9 have been associated with the end of the thoracic series; Hoxc5 and Hoxc6 with the cervical-thoracic transition; and the Hox10 members with the initial segments of the sacrum. The expression of Hoxc6 was further linked with the cervical-thoracic transition and the innervations of the pectoral appendage (Manzanares et al. 2000, Manzanares and Krumlauf 2001, Popodi and Raff 2001).

Therefore Hox genes from many species, as insects and small animals, have been extensively used as model to study the early mammalian embryonic development and their abnormalities, because failures in Hox-gene expression at many different levels cause severe pathological aberrations, as the observed by HOXA10, HOXB3 and HOXB4 in the hematopoietic system (Kappen 2000).

Paralogous members of the hox family as Hoxa9, Hoxb9, and Hoxd9 participate in the mammogenesis at embryonic stages and in adult mouse during pregnancy and after

parturition, also mammary gland expresses Hoxc9 transcripts. This is an example of a similar function observed in the expression of paralogous member of this family. Also their mutations affect milk releasing (Chen and Capecchi 1999).
Thus Hox-proteins work in the process of transcription like factors activating or repressing the expression of target genes being classified as transcription factors. On this form they participate directly or indirectly in the body structural development appearing as a chain of processes. But they are not only expressed in the embryonic development (Lemon and Tjian 2000).

2.2 Cdx proteins and functions in embryonic development

2.2.1 Cdx proteins and functions

In the study of the Homeobox family, it has been needed to pay special attention in a related group, the caudal type homeobox transcription factors (caudal homologues; Cdx1, Cdx2 and Cdx4), which essentially take a central function in the transcription (Chawengsaksophak et al. 2004, van den Akker et al. 2002).
Hu et al. (1993) isolated and characterized the murine Cdx1 gene; soon later James et al. (1994) characterized the Cdx2 gene; and Charité et al. (1998) confirmed that the Cdx1 and Cdx2 genes control the homeobox gene expression in vertebrates. They are characterized for their separated position in the genome showing to have a typical expression pattern, from a caudal to a posterior location at the beginning of the embryonic development. So they are temporally expressed in the development.
Cdx´s are involved in the anteroposterior (AP) patterning of many organisms differing in embryogenesis patterns with widely divergent body plans and forms of development. But in mammals Cdx´s seem to have similar patterns of expression (Chawengsaksophak et al. 1997, 2004, Lohnes 2003).
Cdx gene products in vertebrates can directly regulate other Hox-gene members (Charité et al. 1998, Chawengsaksophak et al. 2004), for this the CDX binding sites play a central role, supporting the idea that Cdx´s are imperative in Hox-gene expression by domains limited at Cdx´s expression levels. These because positional information is proportioned directly to the Hox-genes, acting through such Cdx binding sites normally located in their enhancers or regulatory regions. Thus Cdx members

activate transcription, when its DNA binding elements take place in an enhancer position in the promoters, also having antiproliferative effects.

Cdx members stimulate differentiation and expression of specific genes containing such elements and not only by Hox-genes. This has been similarly observed at nuclear level by its interaction with the amino-terminal domain of CBP gene (CREB-binding protein). CBP may modulate the function of Cdx2 on complex promoters, influencing cellular differentiation and development (Lorentz et al. 1999).

Cdx proteins are gradually produced in the embryo development and are upstream regulators of the Hox-genes expression pattern (Charité et al. 1998, Gaunt 2001). Rather Cdx and Hox genes are elements of a gene network in hierarchical cascades forming the Hox-gene code or spatial map (Chawengsaksophak et al. 2004). The CDX gradient activity is a function of the regulatory elements interacting with its promoters, suggesting the multimerization of regulatory elements (Tabaries et al. 2005). So Cdx expression regulates Hox-gene expression at its initiation phase. Therefore for the correct expression of Hox-genes, the amount of Cdx binding sites plays a crucial role, given that modification in the number of these sites influences the Hox-genes expression, as by others target genes (Charité et al. 1998, Gaunt 2001). Consequently Cdx members conduct the activity of the Hox-gene code, as direct regulators and probably directing the posterior genomic information (Lohnes 2003). Feedback inhibition and cross-inhibitory regulation have been characterized among Cdx members (Chawengsaksophak et al. 2004).

Lorentz et al. (1999) reported the relation between cAMP and Cdx2 pathway. For the histone acetyl transferase functions of CBP are required pituitary-specific factors (Pit-1), which normally are stimulated by cAMP or growth factors. Pit-1 activation by both ways depends on distinct domains of CBP. Thus Pit-1, as a member of the POU-domain proteins group and members of the homeodomain family of proteins, participates in the development and homeostasis by acting in response to signal-translation pathways to either repress or activate the expression of specific genes.

On another form, cAMP amount is directly related with glucose quantities in the cellular medium. This is a possible way as the energetic metabolism could be indirectly involved in the gene expression activation of Cdx or Hox-genes at the early embryonic development (Cantile et al. 2005, Karp 1999, Lodish et al. 2000, Lorentz et al. 1999, Xu et al. 1998).

Lickert et al. (2000) have shown, through the Wnt gene family stimulation in mouse ES cells, that Wnt signalling pathway directly regulates Hox-gene expression by inducing Cdx members mRNA and protein. In mice, Wnt genes are expressed at early development, so Wnt3a transcripts have been detected from 2-cell stage embryos, representing a maternal or embryonic transcription, with a consistent expression detected during blastocyst formation. Both Wnt3a and 4 genes are expressed at precompact 4 to 8 cell stages in mice (Lloyd et al. 2003).

Hsieh et al. (2002) characterized Wnts and Frizzleds (Fz) expression at specific stages as at follicular development levels in the rodent ovary. Wnt ligands and Fz G protein-coupled receptors impact cell fate, including embryonic development of the ovary, suggesting multiple functions for this signalling pathway in such tissue as the found by Pitera et al. (2001) for HOXD group. The interaction among families of genes is well recognized in the Hox-gene expression, as is the participation of the Wnt and Fgf signalling cascades (Chawengsaksophak et al. 2004, Lohnes 2003).

2.2.2 Cdx proteins and embryonic development

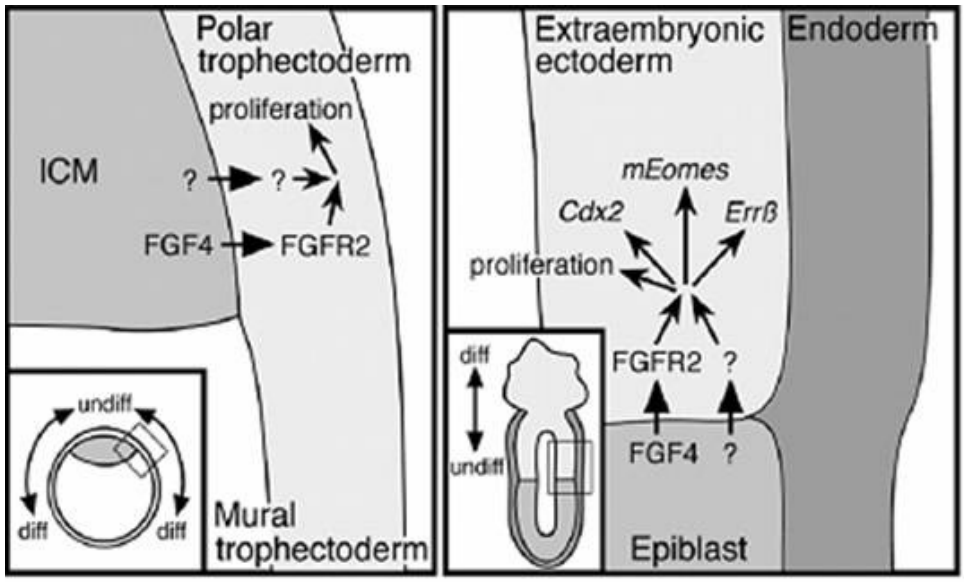
Frequently, it has been assumed that Cdx gene members are expressed at first time by gastrulation and subsequent periods, also by the embryonic anteroposterior patterning. It is known that intestinal differentiation at early development begins late in the embryonic life with the transition from endoderm to an intestinal epithelium, where Cdx2 and Cdx1 regulate the transcription of intestine specific genes and cellular differentiation and proliferation. Still Cdx members are constantly expressed in the intestinal epithelium along the life (Charité et al. 1998, Ehrman and Yutzey 2001, Mallo et al. 1997, Meyer and Gruss 1993, Partanen et al. 1998, Silberg et al. 2000, Imakawa et al. 2004).

Nevertheless, Adjaye et al. (1997, 1999) revealed for the first time transcripts of these genes to be expressed in preimplantation stages, even in immature or mature oocyte, as well as others Hox-members or from the different related groups as exposed above. And Ponsuksili et al. (2001b) reported for the first time the Cdx1 and Cdx2 gene expression in cattle oocytes and preimplantation stage embryo among some others Hox-genes.

Some years before of such discoveries, Beck et al. (1995) detected a possible role of the Cdx2 gene in extraembryonic membranes, this to be expressed in the mouse embryo

and placenta (Goldin and Papaioannou 2003). After nearly a decade in 2004, Imakawa et al. shown that Cdx2, as Eomes other Hox related gene, are necessary for TE differentiation of blastocysts and for subsequent implantation, since homozygous mutant mouse conceptuses of Cdx2 or Eomes genes show similar phenotype (see figure 2.2).

A)



B)

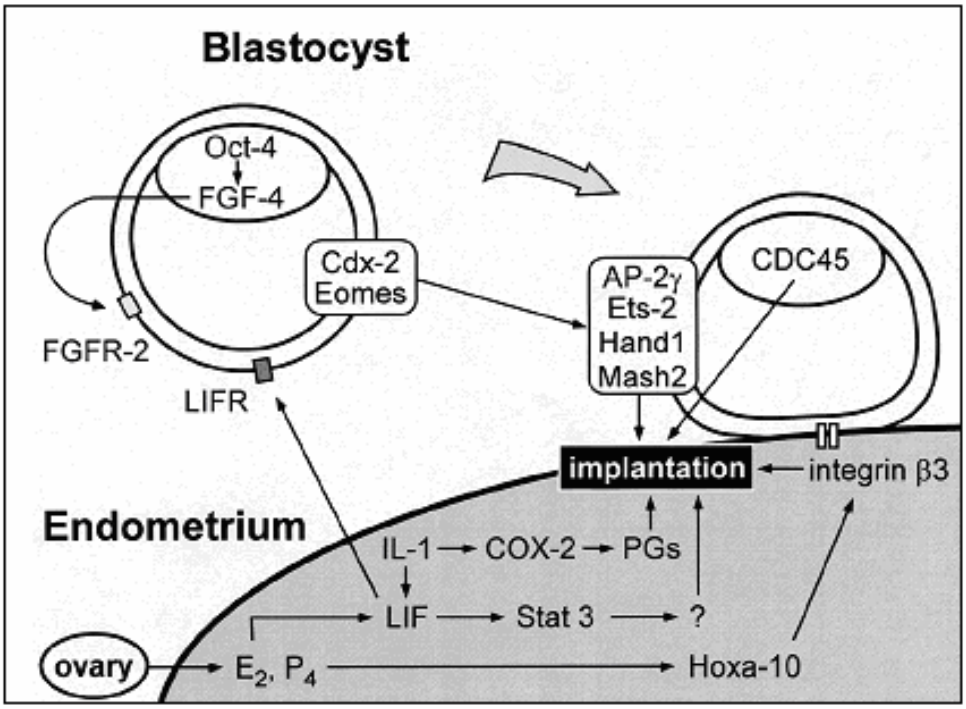


Figure 2.2 A) Expression pathway of genes at the trophectoderm differentiation (Goldin and Papaioannou 2003, Roberts et al. 2004). B) Mouse model for molecular expression pathway at early implantation (Imakawa et al. 2004).

Early blastocyst cells differentiate into ICM (see figure 2.2), where Oct-4 is expressed inducing FGF-4 expression to maintain pluripotency. FGF-4 influences trophoblast cell proliferation through its FGFR-2 receptor, thus trophoblast cells express Cdx2 and Eomes as transcription factors, inducing a chain of transcriptions as AP-2, Ets-2, Hand1 and Mash2 to the proper implantation. CDC45 influences ICM cell proliferation by replication of DNA during the process.

In the endometrium, ovarian estrogens (E2) induce LIF cytokine, activating Stat3 and resulting in the initiation of a cascade of expressions to the uterine preparation for implantation. Serum E2 and progesterone (P4) modulate directly Hoxa-10 transcription for cell adhesion by up-regulating the integrin β3 subunit (figure 2.2). Through leptin and OB-RB comes the activation of IL-1 expression inducing COX-2, and then could be produced and released PGs. Also IL-1 expression induces LIF cytokine sending a message to the blastocyst through LIF receptors (Imakawa et al. 2004). So Cdx members appear to be pivotal for implantation, taking the control of the lineage-restricted expression of Oct-4 and Nanog at morula stage (Tamai et al. 1999, Ralston and Rossant 2005).

On another way, Hinoi et al. (2002) observed in cancer cells like-model, that Cdx2 overexpression increase E-cadherin protein and mRNA levels. Using a similar model, Keller et al. (2004) observed the induction of cell adhesion by Cdx1 and Cdx2 being inhibited by blocking E-cadherin activity. So it could be assumed that Cdx1 or Cdx2 expression induce an E-cadherin dependent adhesion in colon cells. Also LI-cadherin and claudin-2 are targets of Cdx2, well implicated in cell adhesion.

Despite of the previously observed by Lorentz et al. (1997), who did not find measurable changes in cell adhesiveness or morphology in intestinal epithelial cells, E-cadherin was slightly more expressed (1.7-fold) in Cdx2 overexpressed cells, and mRNA level, but not for b-catenin. Anyway could be supposed that Cdx1 and Cdx2 have the capacity to induce cell-cell adhesion and adherens junctions being $Ca2^{+}$ dependent (Lorentz et al. 1999).

E-cadherin cell-cell adhesive activity has been associated to the formation of TE and ICM. Larue et al. (1994) found that null mutant mouse embryos for E-cadherin gene fail to form a TE epithelium. E-cadherin is initiated for adhesion cell at compaction, being critical regulating the orientation cell transformation to ICM and TE differentiation by

rodents (Kwong et al. 2000). E-cadherin has been identified and studied in bovine early development and implantation having a similar pattern of expression (Adjaye et al. 1999, Paria et al. 2001, Rizos et al. 2004). In the last time, Niwa et al. (2005) have observed that Cdx2 expression directs nuclei and surface expression of a placental cadherin, a TE marker also known as Cadherin3 (Cdh3).

In cancer cells like-model, stimulation of TGF-ß1 induced E-cadherin and EGF. TGF-ß1 synergistically decreases E-cadherin expression, but the inhibition of the mammalian target of rapamycin kinase (mTOR) by rapamycin, induces E-cadherin expression despite the presence of high TGF-ß1 (van der Poel 2004). Rapamycin-treated IEC-i-K-Ras cells displayed spindle morphology; thus the expression of E-cadherin was significantly reduced, and the junction localization of E-cadherin was completely lost (Shao et al. 2004). Such interactions are remained to be clarified in early embryonic development, since Cdx2 is expressed in TE (Chawengsaksophak et al. 2004), and now has been recognized that Cdx2 could be early expressed, as done by many other transcription factors, for a proper cell differentiation at periimplantation (Imakawa et al. 2004, Ralston and Rossant 2005, Strumpf et al. 2005, Tamai et al. 1999).

2.2.3 Loss of Cdx proteins in implantation and further development

Drummond et al. (1997) have reported the loss of implantation in Cdx2 homozygous knock-out embryos of mice. Loss of Cdx2 at early embryonic development could cause embryonic death, as observed by Cdx2 homozygous mutants dying at periimplantation (Beck et al. 1995, Chawengsaksophak et al. 1997). Cdx4, but not Cdx1, seems to be able to induce TE differentiation and participate together with Cdx2 in a similar form (Niwa et al. 2005).

Early gastrulation in homozygous mutant embryos appears as normal (Chawengsaksophak et al. 2004). And Cdx2 null mutant blastocysts continue living in the uterus immediately before implantation, but they can not be right detected after implantation period indicating that Cdx2 deficient blastocysts die by failing to the successfully mother-embryo crosstalk, as many developmental effects of improper Hox-gene expression caused by the milieu as previously mentioned (Houle et al. 2000, Deschamps and van Nes 2005).

During later development of the primitive streak are detectable the disturbances, so the first defects are related to abnormalities of extra embryonic mesodermal development. Also Cdx2 null mutant fails to form the chorion-allantoic placenta, because a deficient allantoic development. The posterior part of the streak in mutants produces a defective extra-embryonic mesoderm that could cause an abnormal vasculogenesis of the yolk sac (Chawengsaksophak et al. 2004).

A function dropping of Cdx members could cause homeotic transformations, which are apparent late in the embryonic development. Even in adults could cause health problems, as observed by these Cdx2 heterozygous mutants (Subramanian et al. 1995, Chawengsaksophak et al. 1996, 1997, 2004). Cdx heterozygous mutations cause such homeotic-like transformation, accompanied by changes in the distribution of Hox-gene expression, causing axial truncation, even homeotic alterations along the body axis and posterior shifts in Hox-gene expression (Isaacs et al. 1998, Subramanian et al. 1995, van den Akker et al. 2002). A Cdx2 deficiency produces an anterior homeotic alteration in the specification of midgut endoderm showing that Cdx genes, like a group, exert the homeotic function of them self, being phylogenetically related to other Hox-genes (Chawengsaksophak et al. 2004).

2.3 Association of the homeobox gene family, nutrition and reproduction to developmental important pathways

Early the homeobox gene family was studied as an example of intrinsic cell factors establishing or maintaining the cellular identity at the early embryonic development, as well as in some adult tissues as in this case the gut. Also in the cellular identity play an important role other cell factors defined as extrinsic regulators, as are the growth and differentiation factors, extracellular matrix components and even other cell factors. So extrinsic and intrinsic modulators work together to regulate a particular lineage pathway directing the development. Often members of the former can influence latter transcription factors, which similarly could influence other transcription factors (James and Kazenwadel 1991, James et al. 1994).

Thus Adjaye et al. (2005) explained that during early embryogenesis, the specification and proper arrangement of new cell types require the coordinated regulation of gene

expression and precise interactions between adjacent cells. And these morphogenetic changes depend on the interaction of extra cellular ligands with their receptors.

In the past ten years, it was well characterized that oocyte developmental competence is directly related to the metabolic and hormonal condition of the cow (Armstrong et al. 2001, Armstrong et al. 2002, Webb et al. 2004), which are mediated for her nutritional or physiological condition and all factors inducing changes in the ovarian activity. Such situation is also observable by changes in growth factors locally or externally produced affecting oocyte maturation and developmental competence or quality (figure 2.3). This has been thought, because the ability to develop a blastocyst is directly related to intrinsic conditions carried by the oocyte (Lonergan et al. 2003a, b, Webb et al. 2004, Rizos et al. 2002a, b, c). Furthermore, the establishment of the oocyte population in the fetal ovary and oocyte recruitment from the primordial germ cells (PGC) could be affected by the nutritional or physiological condition of the cow, and any environmental effect at this moment will affect the oocyte pool reducing the future fertility of the female (De Felici 2001).

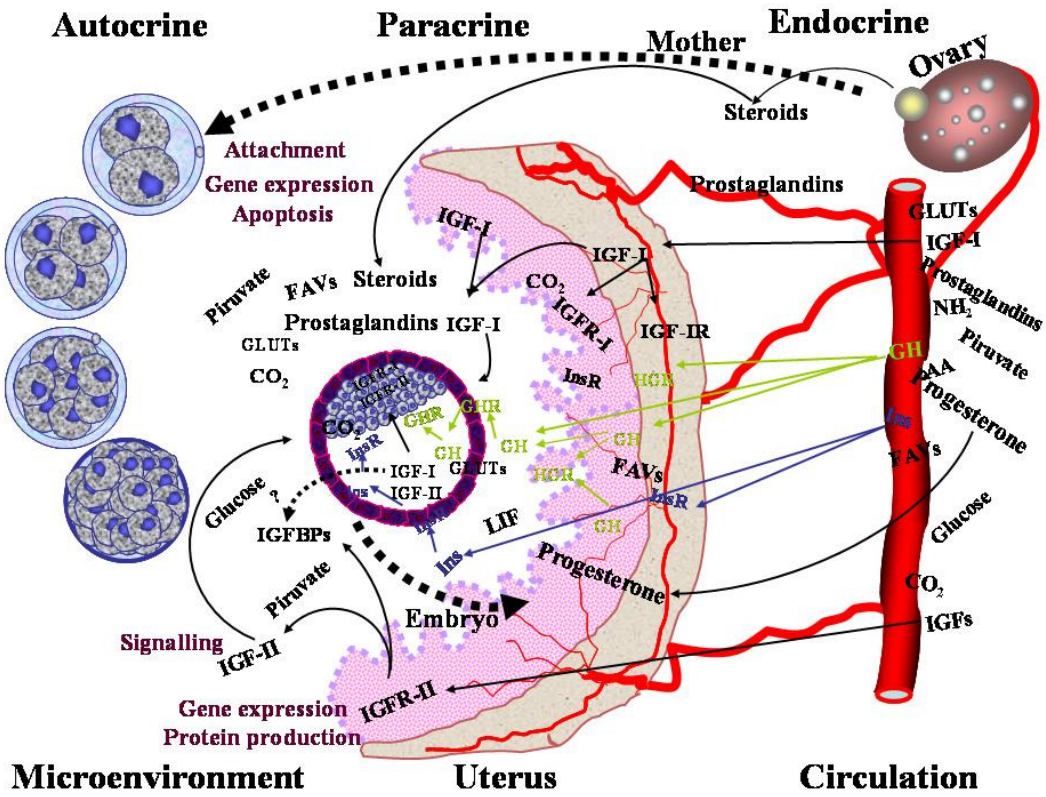


Figure 2.3 Embryo maternal communication and embryonic microenvironment (adapted from Butler 2003).

Many pivotal genes as E-cadherin are expressed in the proximal extra-embryonic mesoderm that contains the PGC precursors. Cell-cell interaction mediated by E-cadherin is crucial for these precursors to be allocated to the germ cell lineage. High levels of E-cadherin are expressed in the epiblast, but not in the mesoderm. These come together with the induction of cell adhesion by E-cadherin activity and the possible interaction of Cdx1 or Cdx2 (Keller et al. 2004).

Niwa et al. (2005) observed that induced overexpression of Cdx2 or repression of Oct3/4 directs the morphological differentiation of TE in a very similar form, having comparable efficiencies of induction for TE differentiation. Furthermore, they found the expression of the placental cadherin Cdh3, as a TE marker induced by Cdx2, but not by Oct3/4 induction. On another form, they suggested that for the differentiation of the TE, the expression of Cdx2 is sufficient and that in trophoblast stem (TS) cells, this gene has a unique function being essential for TS cell propagation. But Eomesodermin (Eomeso), induce differentiation as efficiently as Cdx2, and in absence of this gene, showing an overlapping function with Cdx2. Also Cdx4 seems to be able to induce TE differentiation, but Cdx1 has not been yet observed to have such effect as previously mentioned.

Imakawa et al. (2004) explained very well a succession of molecular events in the embryonic ICM and trophoblast cells establishing the maternal uterine crosstalk during the implantation process. Disruption of one pathway, even in the expression of only one gene being pivotal in the pathway, may result in implantation failure, as has been observed by homozygous mutants of Cdx2, given that such pathways perhaps are interconnected. Consequently interruptions of any pathway will lead to developmental failures related with such events. As Cdx2, other genes as Eomeso, Hand1, Esx1, Dlx3, Psx1, Ets2, and Errb, could be used as markers in the TE differentiation; in which Cdx2, Eomeso and Oct3/4 and their regulatory interactions are determinants for the proper TE and ICM differentiation (Niwa et al. 2005).

Paria et al. (2001) published some genes involved in preimplantation and implantation in mice as E-cadherin, FGF-4, erbB1, a-catenin, IGF-I, Wnt7a, COX-2, ERa, PR, LIF, IL-11Ra IL-11, Hmx3, Hoxa-10, Hoxa-11, cPLA2, CSF-1, most of them have been already analysed in bovine embryo at different stages, ovary and uterus at different time of the oestral cycle. Also some of them as many others are pivotal in a pathway as

cadherins, FGFs, IGFs, Wnts. Pilon et al. (2006) have proposed that to reveal information of signalling pathways involved in the expression of Hox genes, Cdx members appear as interesting candidates. Cdx members could mediate the effects of Wnt3a signalling; for example exogenous Wnt3a can regulate Cdx4 expression in mouse embryos, because Cdx4 promoter responds to Wnt signalling, effect mediate by a putative LEF/TCF response elements. So, as early was observed by Cdx1, Cdx4 is a direct target of the canonical Wnt pathway. Subsequently, Cdx members have been used to relate the signals of RA, FGF, and Wnt pathways regulating the Hox-gene code (Lohnes 2003).

Therefore, an intercellular signalling through enhancer sequences, promoters and other elements within some Hox complexes regulate the activation of the Hox-gene expression (Partanen et al. 1998, Karp 1999, Silberg et al. 2000). Houle et al. (2003) explained that their activation and regulation is controlled by different extern and intern factors as retinoic acid (RA), peptide like growth factors (PGFs), fibroblast growth factors (FGFs), activin family members, Wnt family signalling, and Cdx genes or other elements of the same homeobox family, even perhaps exist a own auto regulation, as observed in the Cdx1 gene in an autoregulatory loop, dependent on Cdx1 by itself (Béland et al. 2004).

Hence RA derived from vitamin A appears to be a potent inducer of development by Hox gene expression, as by PGFs, FGFs, Wnt3a and activins shown to alter the level and pattern of expression of many Hox genes. Until now it is well recognized that Hox gene expression appears to be regulated by different molecular mechanisms and environmental signals very imperative for the maintenance or restriction of a proper Hox expression (Trokovic et al. 2003). In 2001, Pitera et al. found a dosedependent increase in Hoxb1 as other Hox genes as Hoxa1 and Cdx group members by a responsiveness to exogenous RA through RA receptors (RARs and RXRs) expression as well as RAR elements (RARE) showing a retinoid-dependent expression (Allan et al. 2001, Houle et al. 2003).

Around implantation has been demonstrated that IGFs/insulin, Wnts, and FGFs pathways control embryo growth, implantation, elongation, differentiation. Also such pathways induce outgrowth of different organs and sections of the body, as the limb buds or during tooth morphogenesis. Their activity remains during the whole embryonic

development to complete the growth on a similar way (Capdevila and Izpisúa-Belmonte 2000, Dealy and Kosher 1996, Kettunen and Thesleff 1998).

Tesfaye et al. (2003) found a transiently expression of eIF-1A (eukaryotic translation initiation factors) associated to the embryonic genome activation in bovine embryos at the 8-cell stage developed in vitro. This eIF member coincides with the major activation of embryonic genome; this has been used as an expressed endogenous marker of genome activation in mouse and bovine embryos (De Sousa et al. 1998a, b, c). Later Oropeza et al. (2004) suggested that IGF-I could favorably change environmental conditions in the follicle promoting the transcription of Glut-1 and eIF-1A later during the genome activation. So the embryo is prepared for an efficient protein synthesis and glucose metabolism that seems to be essential for the activation of developmental related pathways. Protein translation is controlled by phosphorilating eIF members and ribosomal proteins through mTOR pathway. Thus translation is tightly regulated by the availability of nutrient in the cell, where mTOR, as a censor, coordinates the production of proteins (Du et al. 2005).

Aoki et al. (2003) after researching in mutant mice and colon cancer, conclude that CDX2 is an upstream regulator of mTOR transcription in cancer cells as by may genes as p21, WAF1, CIP1 (Bai et al. 2003, Hinoi et al. 2002, 2005). Also the tumor suppressor adenomatous polyposis coli and Cdx proteins could regulate expression of retinol dehydrogenases (Jette et al. 2004). But a lot of these interactions remain to be found in healthy cells as well as in early embryonic development.

After gastrulation the outgrowth and patterning of limb bud development are dependent on reciprocal interactions between a thickened cap of epithelium at its distal apex (Dealy and Kosher 1996). In the apical ectodermal ridge (AER) and the primary mesoderm of the limb bud have been implicated PGFs, IGFs, insulin and members of the FGF family for the limb fate. At this level IGFs and its receptors are expressed by the subridge mesodermal cells of the limb bud growing out in response to the AER. As a result Dealy and Kosher (1996), suggested that neither IGF-I, insulin nor FGFs induce expression of the Hox gene family members, as Msx-1, in the subapical mesoderm of limb buds. Although they have observed that FGFs, but not IGF-I or insulin, maintain Msx-1 expression in limb bud, and present implications to the relationships among the wl and ll gene families, IGF-I/ insulin system, as FGFs, Msx-1 and Msx-2 or another Homeobox gene, working together in the regulation of limb outgrowth. On this form,

limbs are induced in the embryonic flank at specific positions involving evident combinations of Hox-genes expression (Kettunen and Thesleff 1998, Capdevila and Izpisúa-Belmonte 2000).

All these factors and their relationship with nutrition and cell function are really interesting for the understanding of the ART (figure 2.4) (Thompson et al. 2002). This appears as an option on the lookout for a solution to disorders that are apparent in the offspring, even reflected in the adult health, as is the condition by the large offspring syndrome (LOS) or some developmental well characterized aberrations in adult beings (Lazzari et al. 2002, Young et al. 1998).

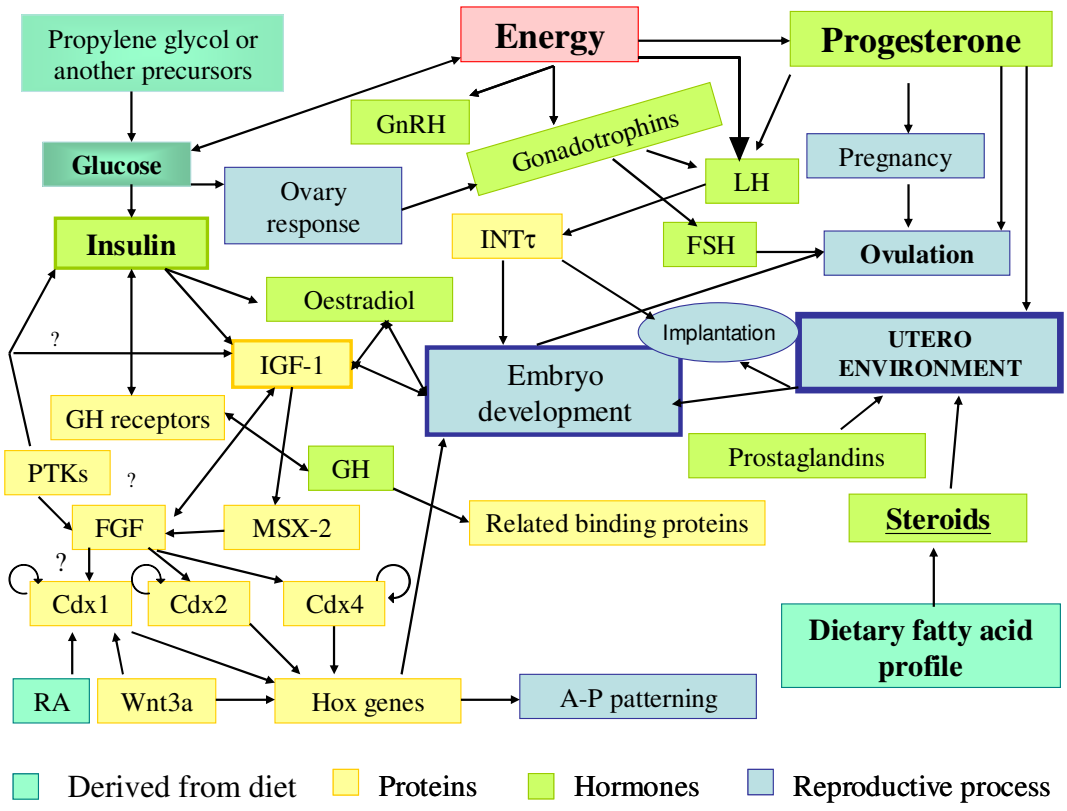


Figure 2.4 Possible relationships among homeobox gene code, nutrition and reproduction (Dealy and Kosher 1996, Formigoni and Trevisi 2003, Lohnes 2003).

According to the information above presented, the characterization of the expression pattern of these transcription factors being pivotal for important events in the oogenesis and early embryonic development could be imperative to improve ART, as in vitro maturation and production of oocyte and embryo respectively. These Cdx and other

Hox members, playing essential roles for a proper implantation and further bovine development, have a high association with functional and morphological phenomena in adult tissues and organs. Those genes have not been well characterized until now. For that reasons our aim has been established to do a molecular examination of these members of the homeobox gene family through the analysis of the genetic expression by means of RT-PCR technologies and the quantitative mRNA abundance by the real-time PCR system.

3 Materials and Methods

3.1 Materials

3.1.1 Materials for molecular analysis

Chemicals, biological materials, instruments and softwares that have been used, will be presented in the following sections. Chemicals and biological materials acquired from different commercial laboratories are presented in the section 3.1.1.4:

3.1.1.1 Reagents and media

Name	Composition	Volumen/ Weight
Ampicillin:	Ampicillin	10mg
	Water added to	1ml
	(Millipore® Milli Q was used)	
10% APS:	Ammonium peroxide disulphate	10gr
	Water added to	100ml
Binding buffer (50 ml):	Tris HCl (1 M pH 7.5)	1.0 ml
	LiCl (5 M)	10.0 ml
	EDTA (0.005 M pH 8)	20.0 ml
	Water added to	50.0 ml
Blue dextran buffer:	Blue dextran (50.0 mg/ml)	1.0 ml
	Ethylene diamino tetra acetic acid (EDTA) (186.1 mg/ml)	50.0µl
	Formamide (Roth)	5.0 ml
Capacitation medium (50 ml):	Sodium chloride	0.2900 g
	Potassium chloride	0.0115 g
	Sodium hydrogen carbonate	0.1050 g
	Sodium dehydrogenate sulphate	0.0017 g
	HEPES	0.1190 g

	Magnesium chloride $6H_2O$	0.0155 g
	Calcium chloride	0.0145 g
	Sodium lactate solution (60%)	184.00 µl
	Phenol red solution (5% in D-PBS)	100.00 µl
	Water added to	50.00 ml
Culture medium		
(CR1aa- 50 ml):	Hemicalcium lactate	0.0273 g
	Streptomycin sulphate	0.0039 g
	Penicillin G	0.0019 g
	Sodium chloride	0.3156 g
	Potassium chloride	0.0112 g
	Sodium hydrogen carbonate	0.1050 g
	Sodium pyruvate	0.0022 g
	L-Glutamine	0.0073 g
	Phenol red solution (5% in D-PBS)	100.0 µl
	Water added to	50 ml
dNTP solution:	dATP (100 mM)	10.0 µl
	dCTP (100 mM)	10.0 µl
	dGTP (100 mM)	10.0 µl
	dTTP (100 mM)	10.0 µl
	Water added to	400.0µl
DEPC-treated water:	Diethyl pyrocarbonate (DEPC)	1ml
	Water added to	1 l
	Incubate for 16-20 hr at 37°C and autoclaving	
D-PBS (Sigma):	Dulbecco`s phosphate buffered saline	g/l
	Magnesium chloride (anhydrous)	0.04683
	Potassium chloride	0.2
	Potassium phosphate monobasic (anhydrous)	0.2
	Sodium chloride	8.0
	Sodium phosphate dibasic (anhydrous)	1.15
Epinephrine solution:	Sodium disulphate	0.0400 g
	Epinephrine	0.0018 g
	Water added to	40.0 ml

FA agarose gel (1.2 %):	Agarose	1.2 g
	10X FA buffer	10 ml
	RNase free water (DEPC-treated water)	90 ml
	EtBr	1 µl
FA buffer (10 X):	MOPS (200 mM)	41 854 g
	Sodium acetate (50 mM)	4 102 g
	0.5 M EDTA (pH 8.0) (10 mM)	20 ml
	Water added to	1000 ml
	Adjust pH to 7.0 and autoclaved	
FA gel running buffer (1X):	10 X FA buffer	100 ml
	37% (12.3M) formaldehyde (FA)	20 ml
	RNase free DEPC-treated water	880 ml
Fertilization medium (FM-50 ml):	Sodium chloride	0.3300 g
	Potassium chloride	0.0117 g
	Sodium hydrogen carbonate	0.1050 g
	Sodium dehydrogen sulphate	0.0021 g
	Penicillin G	0.0032 g
	Magnesium chloride $6H_2O$	0.0050 g
	Calcium chloride	0.0150 g
	Sodium lactate solution (60%)	93 µl
	Phenol red solution (5% in D-PBS)	100 µl
	Water added to	50.0 ml
Hypotaurine solution:	Hypotaurine	0.0011 g
	Sodium chloride solution (0.9%)	10.000 ml
IPTG solution:	IPTG	1.20 g
	Water added to	10.0 ml
LB-agar plate:	Sodium chloride	8.0 g
	Pepton	8.0 g
	Yeast extract	4.0 g
	Agar-agar	12.0 g
	Sodium hydroxide (40 mg/ml)	480.0 µl

	Water added to	800.0 ml
LB-broth:	Sodium chloride	8.0 g
	Pepton	8.0 g
	Yeast extract	4.0 g
	Sodium hydroxide (40 mg/ml)	480.0µl
	Water added to	800.0 ml
Lysis buffer:	Igepal	0.8 µl
	RNasin	5µl
	DTT	5µl
	Water added to	100 µl
Millipore® Milli Q sterilized water:	Millipore® Milli Q water sterilized in an air incubator at 120°C for 1 hr, filtered through a 0.22 µm microcelulose syringe filter (ROTH), aliquoted in 0.2 ml or 1.5ml microcentrifige tubes and irradiated by UV lamp for 60-90 min.	150µl or 1000µl pro tube
Modified Parker medium (MPM):	Sodium hydrogen carbonate	0.080 g
	HEPES	0.140 g
	Sodium pyruvate	0.025 g
	L-Glutamine	0.010 g
	Gentamycine	500µl
	Medium 199	99.00 ml
	Hemi calcium lactate	0.06 g
	Water added to	110 ml
10X PCR buffer:	Tris-HCl	100 mM
	KCl	500 mM
	$MgCl_2$	15 mM
	Gelatine (pH 8.3-Sigma-Aldrich-GmbH, product code P 2192)	0.01 %
PHE (hypotaurine - epinephrine) medium:	0.9% sodium chloride solution	16 ml

	Hypotaurine solution 1mM	10 ml
	Epinephrine solution 250mM	4 ml
Physiological saline solution NaCl (0.9 %):	Sodium chloride	9.0 g
	Water added to	1000 ml
Proteinase K buffer:	Proteinase K	200mg
	1X TE buffer	10 ml
1% SDS:	Sodium dodecyl sulphate (SDS)	1g
	Water added to	100ml
	pH 8.3	
SequaGel® sequencing gel:	SequaGel® XR (National diagnostics, USA)	
	SequaGel® complete buffer reagent	30.00 ml
	Dimethyl sulfoxid (DMSO) (Roth)	7.50 ml
	10% APS	400.00 µl
	(National diagnostics, USA)	300.00 µl
TAE (50 x) buffer:	Acetic acid	57.1 ml
	EDTA (186.1 mg/ml)	100.0 ml
	Tris	242.0 mg
	Water added to	1000.0 ml
	pH 8	
TBE (10 x) buffer:	Tris	108.0 g
	Boric acid	55.0 g
	EDTA (186.1 mg/ml)	40.0 ml
	Water added to	1000.0 ml
1X TE buffer:	Tris (1 M)	10.0 ml
	EDTA (186.1 mg/ml)	2.0 ml
	Water added to	1000.0 ml
	pH 8.0	
Washing buffer (50 ml):	Tris HCl (1 M pH 7.5)	500µl
	LiCl (5 M)	1500µl
	EDTA (0.005 M pH 8)	1000µl
	Water added to	50.0 ml

X-gal:	X-gal(5-bromo-4-chloro-3-indolyl-ß-D-galactoside)	50.0 mg
	N,N´-dimethyl formamide	1.0 ml

3.1.1.2 Softwares

ABI PRISM® sequence detection system software	Applied biosystems, Foster city, CA, USA
BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/).	National center for bioinformation (NCBI)
BLAST 2 sequences	NCBI
Base ImageIR™ reference manual version 4 software	LI-COR, Inc., Biotechnology division, Lincoln, Nebraska, USA
Microsoft® Exel 2002	Microsoft corporation 1985-2001
Microsoft® Word 2002	Microsoft corporation 1983-2001
Primer Express® software version 2.0	Applied biosystems, Foster city, CA, USA
Primer 3´ web-software	http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi
Multalin web-software	http://prodes.touluose.inra.fr/multalin/ (INRA, France)
SAS version 8.0	SAS institute inc., NC, USA

3.1.1.3 Equipment

ABI PRISM® 7000 sequence detection system instrument	Applied biosystems, Foster city, CA, USA
Automated DNA analyser (LI-COR 4200)	LI-COR biotechnology, USA
Centrifuge	Hermle, Wehingen
Electrophoresis pool (for agarose gel)	Bio-Rad., Munich
Incubator (BB16)	Heraeus, Hanau, Germany
Incubator (MCO-17AI)	Sanyo, Japan
Laminar flow cabinet microflow	Nunc GmbH & Co. KG, intervet

Laminar flow cabinet Hera-safe instrument	Heraeus, Hanau, Germany
PCR thermal cycler Bio-Rad I cycler	Bio-Rad, USA
PCR thermal cycler (PTC-100^{TM}) programmable thermal controller	MJ Research, Inc. USA
Philips UV box	PHILIPS, Holland
Millipore® Milli Q system	Millipore® Milli Q
Spectrophotometer UV/ visible light (DU-62 series)	Beckman, Munich, Germany
Stereo microscope (SMZ 2B)	Nikon, Japan
Thermoshake Gerhardt	John Morris scientific, Melbourne
UV transilluminator (Uvi-tec) and gel doc system	Uvi-tec, Uni-Equip, Martinsried, UK

3.1.1.4 Manufacturers, chemicals and kits

Manufacturer	Place	Products
Biozym diagnostik	(Hessisch-Oldendorf)	Sequagel XR sequencing gel (National diagnostics) and SequiTherm Excel^{TM} II DNA sequencing kit (Epicentre technologies)
B-Braun	(Hamburg)	Meliseptol®
Bode	(Melsungen)	Bacillotox®
Gibco BRL, Life technologies	(Karlsruhe)	BME (Amino acids), MEM (non essential amino acids), gentamycin, Trizol^{TM} reagent
Intervet	(Boxmeer)	Equine chorionic gonadotrophin (eCG), Human chorionic gonadotrophin (hCG)
Invitrogen	(CA, USA)	Superscript II reverse transcriptase, DTT, 5 x first strand buffer
MWG Biotech AG	(Ebersberg)	Oligonucleotide primers
MWG-Biotech AG	(München)	Oligonucleotide primers
Nunc	(Roskilde)	Four-well dishes, cryotubes
Promega	(Mannheim)	DTT, pGEM®-T vector, RNase free DNase, Ribonuclease inhibitor (RNasin), RQ1 RNase – Free DNase, T4 DNA ligase and 2 x rapid

		ligation buffer
RUW	(A.G. Münster)	Frozen bull semen
Roth	(Karlsruhe)	Acetic acid, agar-agar, ampicillin, ammonium peroxide disulphate (APS), boric acid, bromophenol blue, calcium chloride, chloroform, dimethyl sulfoxide (DMSO), dNTP, ethylene diamine tetra acetic acid (EDTA), ethanol, EtBr, formaldehyde, isopropyl ß-D-thiogalactoside (IPTG), peptone, proteinase K, silver nitrate, sodium carbonate, sodium chloride, SDS (sodium dodecyl sulphate 99%, tris, 5-bromo-4-chloro-3-indolyl-ß-D-galactopyra-noside (X-gal), yeast-extract
Sigma-Aldrich Chemie GmbH	(Taufkirchen)	Albumin, D-PBS (Dulbecco`s phosphate buffered saline), epinephrin, genelute™ plasmid mini-prep kit, hmi-calciumlactat, heparin, HEPES (4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid), hyaluronidase, hypotaurin, igepal, isopropanol, L-glutamin, magnesium chloride, medium 199, mineral oil, penicillin, phenol red solution (5% in PBS), potassium chloride, 10 x reaction buffer, SYBR® Green JumpStart™ Taq ready Mix™, sodium hydrogen carbonate, sodium hydrogen sulphate, sodium lactate solution (60%), sodium pyruvate, streptomycin sulphate, Taq polymerase
SIGMA-ARK	(Darmstadt)	Oligonucleotide primers
STARLAB GmbH	(Ahrensburg)	Thin wall 96 x 0.2 ml skirted microplates
Stratagene	(Amsterdam)	5α DH Escherichia coli competent cells

3.1.2 Biological materials

Our objective was to establish the expression pattern of some genes of the homeobox family in the early embryonic development in cattle, before and after implantation. In other mammalian species a lot of the homeobox family members are expressed in adult tissues. Among those members Cdx1, Cdx2, Cdx4 and the members of the HoxD group are expressed in the posterior gut endoderm during later embryonic development, in the intestinal epithelium after birth, and throughout life time. These were taken as a positive control of gene expression (Beck et al. 1999, Bostrom et al. 2000). In addition, some genes could be expressed in low abundance in some cells, which probably could be a frequent situation in oocyte or embryo cells. Therefore, adult tissues expressing the homeobox family members were analysed first.

3.2 Molecular analysis procedures

In this section, the methods will be described, which were established to the preimplantation and postimplantation development for the mRNA analysis (Adjaye 2005, Adjaye et al. 1997, Wrenzycki et al. 1999, 2000, 2003, Ponsuksili et al. 2001b, 2002, Garcia-Castillo et al. 2002, Tesfaye et al. 2004, Rizos et el. 2004).

3.2.1 Primer design

Here were employed degenerated primers as a strategy to find Hox-gene ESTs as done by Ponsuksili et al. (2001a, b). But the real-time PCR analysis could not be possible to be performed from such ESTs, because such Hox-gene fragments contain the homeodomain (Pfaffl 2001, 2003). For that reason it was necessary to follow other way to get up the specificity of each gene. It is important that the primers to amplify Hox-gene fragments should not have nucleotide homologies with their orthologous genes and primers should be species specific (Pennacchio and Rubin 2001, Andersson et al. 1996). But specially, primers should not have nucleotides homologies among their paralogous genes, on this form they would be gene specific (GS). In our work we tried to achieve bovine gene-specie specific (GSS) primers, with the idea of assuring amplification only in a specific gene of the bovine genome and avoiding possible contamination with

another genome. Therefore, in this study different systems and two softwares were employed to help the design of the primers and achieve the ESTs of interest. So, by designing primers much care was taken to avoid primer dimer formation.

By trying to amplify the 3´ and 5´ ends of the Cdx1 and Cdx2 genes by the BD SMART^{TM} rapid amplification of cDNA ends (RACE) amplification kit (BD Biosciences Clontech). RACE-primers were designed according to the manufactures instructions and using ESTs sequences containing the homeodomain.

Accordingly, another way was to design anchored primers to direct sequencing of a Cdx1 gene-specific PCR product. Such system has had the aim to design primers by analyzing gene-specie specific sequences to amplify a specific gene fragment. But one of both oligonucleotides was synthesized containing an attached oligonucleotide sequence named anchor, which can be amplified by the T7 or the SP6 primers used for sequencing. Such effective system was designed to be used as a strategy for direct sequencing of PCR product by using the 5´ IRD 800 T7 labelled primer to sequence the 800nm detection channel of the LI-COR 4200 series DNA scanning system (LI-COR, Inc., Biotechnology division, Lincoln, Nebraska, USA). Primers were Cdx1 homo for-GAAGGAGTTTCACTACAGCCGT and the Cdx1 homo rev (T7 primer plus CDX1)-**TAATACGACTCACTATAGGG**TCCCACAATCTGGAAACC.

A third way was established by looking for the amplification of *Bos taurus* ESTs with elevated homology between their orthologous homeobox genes. To be precise, genes that have been already identified and sequenced from other species as human, mouse, rat, other rodents, pig, primate, chicken and even fishes or frogs. They were found in the data bank of the National Center for Bioinformation (NCBI) database (http:www.ncbi.nlm.nih.gov) (McGinnis and Madden 2004). By means of the multiple sequence alignment by the Multalin web-software by Corpet (1988) (INRA, France) homologous fragments among different species were found. Homolog fragments longer than 15 nt presenting 95% of homology were identified and took as primer sequences.

By means of such multiple sequence alignment with the hierarchical clustering system among sequences of 3 to 5 different species, primers were designed for each Cdx2, HoxD1, HoxC9, and HoxB9 genes. Sequences were tested for melting temperature (Tm) and complementarities through Primer Express® sequence design software v2.0 developed by Applied biosystems. Primers were tested for amplification to assure the gene- specific condition.

The fourth round was planed to design sequences for GS primers. They were designed from bovine ESTs derived from mRNA and PCR experiments that were sequenced in our laboratory (HoxD4) or from bovine ESTs (HoxB7), this *Bos taurus* sequence by HoxB7 gene with the accession number NM_174342 (gi:31342273) derived from database of NCBI was used to design GS primers (Bostrom et al. 2000). On this round, all pairs of specific primers were designed employing Primer 3´ web software (Rozen and Skaletsky 2000) or Primer Express® sequence design software v2.0. To design optimal pair primers, parameters were chosen to get primers using default set by the software as it was possible.

The last round was planed to design GSS primer sequences from the bovine ESTs found. This was done using Primer Express® software v2.0 (Applied biosystems) to be employed by the quantitative real-time PCR. Parameters were set to get primers with a Tm of 58-60 °C, not 3´ complementarities to avoid primer-dimer formation or other side-product (complementary products), and to amplify a product with a size between 100 to 200bp (Pfaffl 2001, 2003). All according to the recommendations of Applied biosystems for real-time semi quantitative PCR and with these highest amplification efficiencies should be reached (Tichopad et al. 2002). The sequence and product size of all specific primers used by expression analysis are listed in Table 3.2.2.1.

Table 3.2.2.1 Gene-Specie-Specific primers sequence used for gene expression analysis.

Transcript	Strand	Primer sequence	(n)	Tm°
Cdx1	sense	5´-GCGGAAATCAGAGCTGGC	154	58.1
	antisense	3´-GGAGGTGATGTCATGGGCTG		56.9
Cdx2	sense	5´-TGGGCAGCCAAGTGAAAAC	114	58.3
	antisense	3´-TCCGGATGGTGATATAGCGAC		58.5
HoxB7	sense	5´-GCCAGACCTACACCCGCTATC	347	59.6
	antisense	3´-TCTCCTTTTTCCACTTCATGCG		60.2
HoxB9	sense	5´-CGGATCAAACCAACCCCCTC	182	62.3
	antisense	3´-ATTTTCATCCGCCGGTTCT		58.1
HoxC9	sense	5´-ACTGGATTCACGCCCGTTC	206	59.5
	antisense	3´-CATCCTCCGATTCTGAAACCA		58.6
HoxD1	sense	5´-AGCTTCTTCCATTTCATGCTGC	213	59.5
	antisense	3´-TGGAGCTGGAGAAGGAGTTTCA		59.9
HoxD4	sense	5´-ACGGCATGTTCTTTGGTTC	136	54.7
	antisense	3´-CCGACGTGGCGATATTCAAG		60.0

(n) means nucleotides length in product amplified. Tm and complementarities have been determined by Primer Express® software v2.0.

All oligonucleotides were synthesized by SIGMA-ARK or MWG-Biotech AG, received in a lyophilized form and diluted with UV irradiated Millipore® Milli Q water to a 100 micromolar (µM) concentration. It was done according to the reported concentration in nmol, and then diluted to a PCR working concentration of 10 pmol/µl (10µl stock solution + 90µl water). All primers for standard PCR were optimized looking for the better amplification product by agarose gel electrophoresis and primers for real-time semi quantitative PCR were optimized according to the limiting primer concept of ABI PRISM® 7700 system.

3.2.3 Samples collection

3.2.3.1 Tissues for gene expression analysis in adult tissues

To achieve nucleotide sequences from RNA and the gene expression analysis, a method was established according to published literature in human, mouse and rat to amplify and sequence ESTs by using tissues in which the genes are constantly expressed, as explained above. On this form, 4 fragments of approximately 5 cm^2 of each bovine colon and jejunum were dissected and collected manually from carcasses at slaughter, washed two times in 0.9% NaCl physiological saline solution. The fragments were then briefly sterilized in 75% ethanol, and washed two times in 0.9% NaCl physiological saline solution and immediately were snap-frozen and stored at -80 °C. They were then ready for the total RNA isolation by Trizol® reagent (Gibco BRL, Life Technologies) protocol. That process will be explained below. All tissues selected for RNA isolation, were always snap-frozen immediately by introducing them in liquid nitrogen and stored at -80 °C to avoid the degradation of mRNA transcripts.

For gene expression analysis in the pre- and post- implantation stages, two methods were established. One was the in vitro production of embryos, since in vitro maturation of oocytes until blastocyst stage (days 7 to 9). The second way was the production and recollection of postimplantation stage embryos. After a process of synchronization, bovine embryos between 19 to 45 days post insemination were developed in vivo and collected after slaughter in a local abattoir. One embryo approximately between the 60^{th}-70^{th} days old was collected after slaughter and prepared too. Both procedures will be reviewed in the following sections.

3.2.3.2 Sample preparation for the preimplantation analysis

Bovine oocyte maturation and embryo production were obtained using techniques previously standardized in the laboratory of the research station of the University of Bonn and previously described by Ponsuksili et al. (2001b, 2002), El-Halawany (2003), Tesfaye (2003), Tesfaye et al. (2004), Mamo (2003), and Schneider (2003).

In vitro maturation of oocytes

Ovaries were collected in a local abattoir directly from bovine carcasses in a well established handling-method. Ovaries were placed in thermo flasks containing 0.9% NaCl physiological saline solution with Streptocombin® (50 µl/100 ml) at 35°C. They were transported to the laboratory within 3hr, where immature bovine cumulus oocyte complexes (COCs) were obtained by the well known technique of syringe-needle aspiration. All ovaries were immersed in 70% ethanol to be disinfected and rinsed twice in 0.9% NaCl physiological saline solution. Both solutions beforehand warmed at 35°C. Ovaries were then dried with sterile paper towels and immediately follicles with the size of 2 to 8 mm^3 were aspired by means of syringe (10 ml) and needle (18-20 Gauge).
Follicular fluid was recollected in sterilized 50 ml falcon tubes prewarmed at 38°C to avoid cold-shock and left for 15-20 min to be precipitated. By means of pipetting with a Pasteur pipette formerly polished by flame at the tip and equipped with a suctions latex gum, COCs were recovered from the bottom of the tube to be separated in 90 mm Petri dishes containing modified parker medium (MPM-110ml). And then they were supplemented with 12% heat inactivated oestrus cow serum (OCS) and 10 µg/ml recombinant follicle stimulating hormone (porcine-FSH-Schering, Kenilworth, NJ, USA.). The hormone was prepared earlier and left on a warm-table at 38 to 40 °C. Thereafter, under a stereoscopic microscope (40x) (Nikon, Japan), COCs were washed two or three times using tissue culture medium-119 (TCM-199) (Sigma-Aldrich-GmbH, M-2154) supplemented with 4.43mM Hepes, 33.9mM NaHCO3, 2mM pyruvate, 2.92mM calcium lactate, 55µg/ml gentamicin and 15% heat-inactivated OCS.
Oocytes were separated in 2 groups according to the recommendations of Gwazdauskas et al. (2000), Ponsuksili et al. (2002) and Schneider (2003), but to assure oocyte developmental competence, survival and a superior secretion of sperm

chemoattractants, according to the work of Domínguez (1995), Gwazdauskas et al. (2000). For the first group (excellent), COCs surrounded by an intact zona pellucida, a compacted multiple layers (more than three) of cumulus oophorus cells with homogeneous dark cellular ooplasm and uniformly granulated cytoplasm were selected and matured. Oocytes containing dark homogeneous plasma, exhibiting a loosening or a tiny layer of cumulus oophorus cells (less than three) were classified as a second class, and COCs containing a degenerated cytoplasm or disintegrated surrounding cumulus cells allowing a nude oocyte were discarded.

After selection and rinsing, groups of 50 selected oocytes were transferred to a four wells dish (Nunc, Roskilde, Denmark) containing 400µl TCM-119 - Sigma-Aldrich-GmbH, M-2154) supplemented with 10% (v/v) fetal calf serum (FCS) and 10 ng/ml epidermal growth factor (EGF), previously warmed for 50 to 60 min at 39°C and covered with a drop of mineral oil (Sigma-Aldrich-GmbH). Four well dishes (Nunc, Roskilde, Denmark) were introduced to an air-incubator (BB16-Heraeus, Hanau or MCO-17AI-Sanyo, Japan) prepared to have an air humidified atmosphere with 5% CO_2 at 37 °C where oocytes were matured through 22 to 24 hr in incubation.

After incubation, matured oocytes were selected under a stereomicroscope (Nikon, Japan) to be used for the molecular analysis. It is worth mentioning that only the first group was selected for our work.

In vitro fertilization (IVF)

In our procedure, following maturation, oocytes have been inseminated with frozen-thawed proved bull semen of a German breed, which was previously proved for IVF. Spermatozoids were treated by swim-up technique developed by Parrish et al. (1986, 1988) to separate living spermatozoids having higher fertilization efficiency.

Frozen sperm straws from liquid nitrogen were thawed by introducing in a 39 °C water bath for 10 sec. Straws were dried with tissues handkerchief, washed with 75% ethanol and dried again at air. The straws were carefully cut and sperm was slowly dropped through the wall of a 15 ml falcon PP-Test sterile tube (Cellstar) containing 5 ml prewarmed capacitation medium, supplemented with heparin and buffered with Hepes (Sigma-Aldrich-GmbH), which is a potent sperm or oocyte DNA damage protector of reactive oxygen species (ROS) (Ermilov et al. 1999). Tubes containing spermatozoids

were introduced in an incubator (BB16-Heraeus, Hanau or MCO-17AI-Sanyo, Japan) with humidified atmosphere of 5% CO_2 in air, positioned at 45° of inclination and incubated at 39 °C for 50 min.

By using a sterilized Pasteur pipette, the upper phase of the medium, containing the motile swimming spermatozoids (approximately 0.85 to 1.5 ml), was transferred to a new 15 ml centrifuge tube (Greiner GmbH, Frickenhausen) and centrifuged at 250 RCF/G for 10 min to obtain a spermatozoids pellet at the bottom tube to facilitate its recollection. Supernatant was discarded and then the pellet was washed two times more in 3 ml prewarmed capacitation medium. Adding 3.5 ml prewarmed capacitation medium, surviving spermatozoas were resuspended by pipetting. Before using the sperm for IVF, its motility, viability and quantity were tested by mixing 2µl resuspended sperm plus 198µl double distilled water (1:200) under phase contrast microscopy through 200X zoom on a double grid Neubauer® haemocytometer chamber (Fa. Brand) covered with a planed slide (Tomlinson et al. 2001). The concentration was established at 2 million of spermatozoids per ml, and using an amount of 800 000 spermatozoids per well containing 50 oocytes each one. The sperm amount to be added to each 50 oocytes was calculated using the formula:

800 000 = µl of sperm cells solution / Average of sperm cells in squares X 1000

Four-well dishes (Nunc, Roskilde, Denmark) containing 400µl fertilization medium supplemented with 6 mg/ml bovine serum albumin (BSA), 1 mg/ml heparin, 2.2 mg/ml sodium pyruvate, 10µl of PHE (20µM penicillamin, 10µM hypotaurin, 2µM epinephrine- Sigma-Aldrich-GmbH) and 50µg/ml gentamicin and covered with a drop of mineral oil (Sigma-Aldrich-GmbH) were preincubated for approximately 90 min in 95% air with 5% CO_2 highly humidified at 39°C. Matured COCs were previously washed by gently pipetting two times with fertilization medium in a prewarmed Petri dish and transferred into the preincubated four-well dish to be fertilized. Fertilization was achieved by incubation during 18 to 22 hr in the same conditions as applied for oocyte maturation.

In vitro culture of embryos

After the established time post insemination (hpi), to denude presumptive zygotes of the surrounding cumulous cells and taking out the exceeding attached sperm, were transferred one by one by pipetting to a 15 ml centrifuge tube (Greiner GmbH, Frickenhausen). The tube was previously warmed containing 1 ml culture CR1aa medium (Rosenkrans and First 1994). Medium containing essential amino acids (EAA) and nonessential amino acids (NEA) supplemented with 10 µl/ml of each minimal essential medium (MEM) with NEA and basal medium Eagle (BME) containing EAA as well as 10% OCS.

These tubes were gently mixed up with the finger for 2 min and then the whole amount, containing zygotes, was transferred to a prewarmed Petri dish containing drops of D-PBS (Dulbecco´s Phosphate Buffered Saline; Sigma-Aldrich Chemie GmbH). D-PBS was supplemented with 0.5 mg/ml BSA, 50 mg/ml gentamycin and 36 mg/ml pyruvate (Sigma-Aldrich Chemie GmbH).

Under a stereomicroscope (40x zoom, Nikon, Japan), each zygote was washed two times by pipetting in culture CR1aa medium being selected in groups of 25 zygotes. They were than transferred to the previously prewarmed four-well dishes (Nunc, Roskilde, Denmark) containing 25 ml culture CR1aa medium to be incubated at 39°C in 5% CO_2 and 95% air highly humidified.

Embryos were recovered at each corresponding time (2-cell 38-40 hpi, 4-cell 46-48 hpi, 8-cell 71-73 hpi, 16-cell 98-100 hpi, morula 112-125 hpi, blastocyst 160-175 hpi, about 6 to 7 days post insemination). In vitro cultured oocytes and embryos were washed two times in D-PBS (Sigma-Aldrich Chemie GmbH), transferred to drops of thyroid solution (Sigma), left for 20 sec to destroy the zona pellucida, and then were washed further by pipetting two times in D-PBS and finally transferred with minimal amount of D-PBS into cryo-tubes (Nunc, Roskilde, Denmark or Axygen).

Recovered embryos were lysed by adding 2µl of lysis buffer and directly shacked with the finger at the bottom of the tube and right away introduced in liquid nitrogen for fast snap-chilling and stored in a freezer at –80°C to be used for further molecular analysis.

3.2.3.4 Sample preparation for the postimplantation analysis

Synchronization and insemination of cows

During 12 days, from d-12 to d 0 or first artificial insemination (AI), where day 0 (d0) was defined as the onset of the estrus, cows were injected two times in intervals of 12 hr with prostaglandin $F_2\alpha$ (500µg Estrumate® Cloprostenol, Essex Munich, Germany or Mallinckrodt, Burgwedel, Germany). Between the 9^{th} and 12^{th} day (d-4 to d-1) following treatment to prevent the final dominant follicle suppressive action over follicles to get stimulation, dominant follicle were detected and suctioned through an ultrasound-guided transvaginal follicular aspiration (TVFA) ovum pick up (OPU). 36 hr after follicle ablation, Folltropin-V (porcine FSH; Vetrepharm, Ontario, Canada) was intramuscularly injected each 12hr during 4 days in decreasing doses (daily 8 ml, 6 ml, 4 ml, 2 ml) until 400 mg (20 ml) to initiate superovulation. 2000 IU eCG (Intergonan, Intervet, Boxmeer, The Netherlands) were also intramuscularly injected to stimulate donors. Together with the last two FSH injections 500 mg of $PGF_2\alpha$ (Estrumate; Mallinckrodt or Cloprostenol (500µg) Estrumate®, Essex Munich, Germany) were simultaneously injected in 12hr intervals to induce luteolysis. After 24 hr, the first AI was performed with frozen-thawed proved bull semen of German Holstein Friesian breed. At the onset of estrus 2,500 IU of hCG (Ovogest; Intervet, Boxmeer, Netherlands) were intravenously injected. Two more inseminations at 12hr intervals were performed with the same semen and the same inseminator, whenever the second insemination was defined as the expected time of ovulation.

Sample preparation

From the process of synchronization and AI explained above, cows were tested for pregnancy and slaughtered in a local abattoir between 19 to 45 days post insemination (dpi). Postimplantation embryos were collected at 19, 21, 25, 32, 39 and 42 dpi, separated from placenta, photographed (figure 3.1). Ex vivo recollected embryos were introduced in 50ml falcon tubes and immediately snap-frozen as fast as possible in liquid nitrogen, and stored at -80°C. One embryo, approximately around 60 to 70 days post matting (embryo-fetal transition), was collected after slaughter and kept frozen.

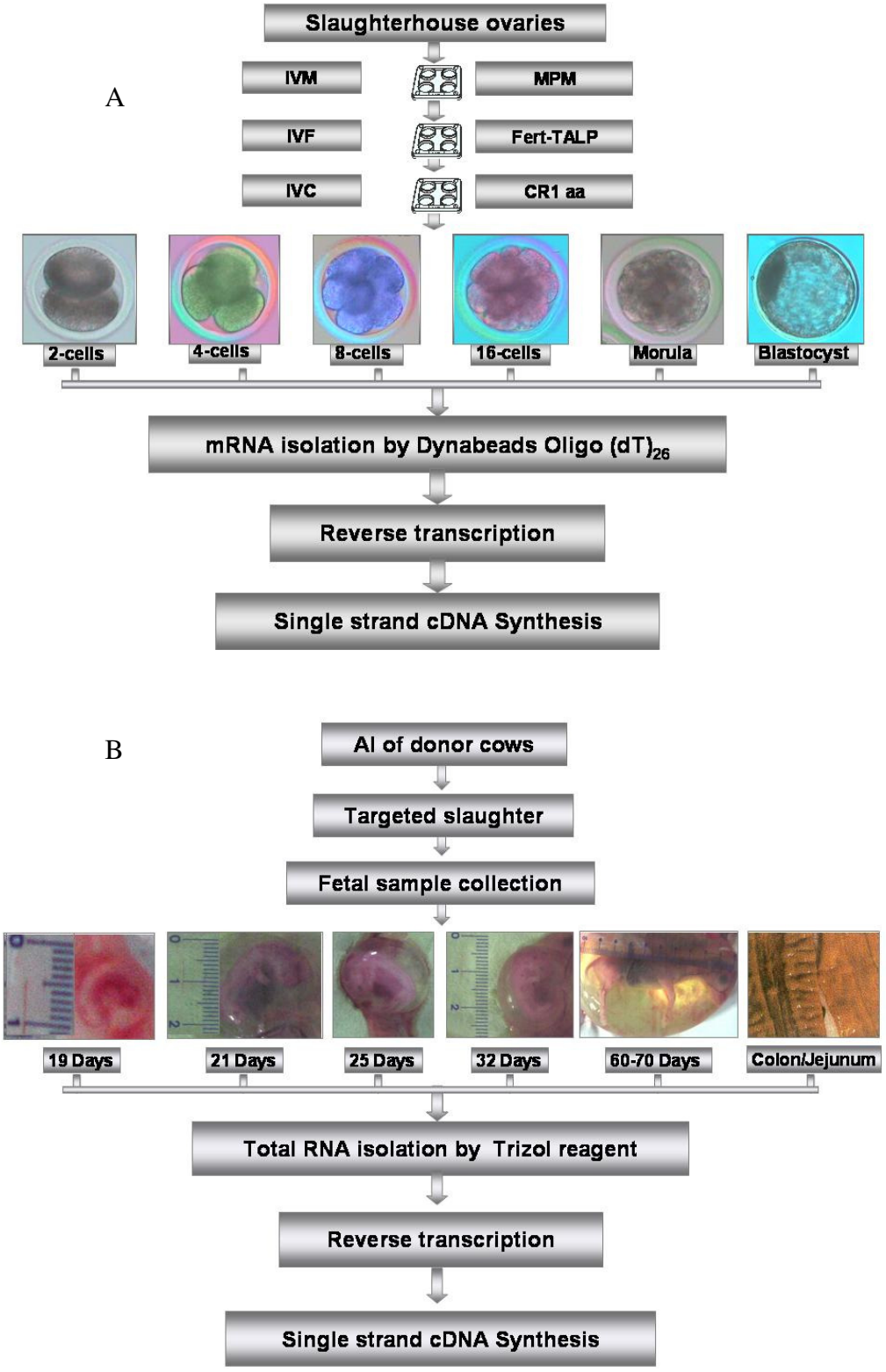


Figure 3.1 A) Sample processing from preimplantation to the real-time PCR quantification system. B) Sample processing from postimplantation to the real-time PCR quantification system.

3.2.4 Isolation of nucleic acids for molecular analysis

3.2.4.1 RNA isolation

To obtain a high quality and a good quantity of RNA, two methods were used. mRNA isolation was done by means of Dynabeads magnetic (Dynal, Oslo, Norway or Life Technologies) for samples containing a small number of cells (oocytes or preimplantation embryos) and the Trizol® reagent (Gibco BRL, Life Technologies) protocol for samples containing a large number of cells (postimplantation embryos, colon or jejunum).

mRNA isolation from preimplantation bovine embryos

By means of Dynabeads Oligo (dT) 25 (Dynal Biotech ASA, Oslo, Norway), mRNA was isolated following the protocol instructions. Prior to thawing lysed-embryo on ice bath, 10µl of Dynabeads Oligo dT 25 (Dynal Biotech ASA, Oslo, Norway) was mixed with 20µl of binding buffer in a new 0.65 ml microcentrifuge tube (Carl Roth GmbH-Mµlti®-Reaktionsgefäße/DNase/RNase-frei) to be cleaned by using the MagneSphere® magnetic separation stand (Dynal Biotech ASA, Oslo, Norway). Approximately 15 sec latter, the liquid phase was carefully discarded by pipetting, and this step was made 2 times. At the end, the Dynabeads Oligo dT 25 (Dynal Biotech ASA, Oslo, Norway) was kept in 20µl binding buffer at room temperature (22–25°C).
40 - 80µl of binding buffer was added to the tube containing thawed, lysed-embryo, mixed by finger-shaking and incubated in a PTC-100^{TM} programmable thermal controller (MJ Research, Inc.) at 70° C for 5 min. During the incubation, the tube containing Dynabeads Oligo dT 25 (Dynal Biotech ASA, Oslo, Norway) was placed onto the MagneSphere® magnetic separation stand (Dynal Biotech ASA, Oslo, Norway) and the liquid was remove by pipetting. The total mix of lysed-embryo was transferred, after 5 minutes, into the Dynabeads and mixed very well by finger-shaking and left at room temperature (22-25°C) for 30 min to allow the binding of the Dynabeads Oligo dT 25 (Dynal Biotech ASA, Oslo, Norway) to the poly A tail of the mRNA in the fluid containing lysed cells.

After the incubation, the tube was placed onto the MagneSphere® magnetic separation stand (Dynal Biotech ASA, Oslo, Norway) for about 15 sec and then the fluid phase was discarded. 150µl of washing buffer was added to the tube, mixed by shaking with the finger and placed onto the MagneSphere® magnetic separation stand (Dynal Biotech ASA, Oslo, Norway) for 15 sec. Using a DNase/RNase free pipette, the liquid phase was carefully discarded. This washing procedure was repeated three times.
13µl of DEPC-treated water were pipetted in the tube, mixed vigorously with the finger and incubated at 95° C for 5 min. A quick and short spin was done with a microcentrifuge (Hermle, Wehingen) at 3,000 RCF/G speed at room temperature to place the mix at the bottom and then the tube was placed onto the MagneSphere® magnetic separation stand (Dynal Biotech ASA, Oslo, Norway) for 15 sec to take out, as much as possible, the clean liquid into a new 0.65 ml microcentrifuge tube (Carl Roth GmbH-Mµlti®-Reaktionsgefäße/DNase/RNase-frei).

Total RNA isolation from postimplantation bovine fetuses, colon and jejunum

Total RNA was isolated by means of Trizol® reagent (Gibco BRL, Life Technologies) such technique is an improvement to the method of Chomczynski and Sachi (1987) and a registered trademarks of Molecular Research Center, Inc. (Nakajima et al. 1988, Simms et al. 1993). Following the protocol according to the manufacturer instructions, products were purified with RNeasy kit (QUIAGEN, Hilde).
Samples, mortar, pestle and scalpel were previously kept at -80°C, during the procedure they were manipulated in liquid nitrogen for tissue sample milling. The mortar was placed on a polystyrene box containing liquid nitrogen to keep the frosty chain. From each sample was cut a bit of approximately 0.5 cm long (like a rice grain) from the intra-abdominal portion of embryos with more than 21 dpi. But for the embryo at 19 dpi the whole embryo was taken. Each sample was carefully pulverized with a pestle into a mortar individually.
In a 2 ml Mµlti®-Reaktionsgefäße tube (DNase/RNase-frei, Carl Roth GmbH) 1ml Trizol® reagent (Gibco BRL, Life Technologies) was added covering approximately 20 to 50 mg of pulverized tissue. Each sample was homogenized with a new syringe and needle and incubated at room temperature for 5 min. Chloroform (200 µl) was added and the tubes were vigorously shaken for 15 sec and incubated for 2 to 3 min at room

temperature, and then centrifuged at 12 000 RCF/G for 15 min at 2 to 8 °C. This step was repeated once more, but with 500µl Trizol® reagent (Gibco BRL, Life Technologies) and with 100µl chloroform, respectively. Because the RNA remains exclusively in the upper aqueous phase, it was transferred to a new 2 ml sterilized Mµlti®-Reaktionsgefäße tube (DNase/RNase-frei, Carl Roth GmbH). Equal volume of isopropyl alcohol was added and incubated for 10 min on ice plate (approximately at 4°C).

Afterwards, samples were centrifuged at 12 000 RCF/G for 10 min at 2 to 8°C. At this moment RNA was visible as a gel like pellet at the bottom of the tube. The supernatant was removed and the pellet was washed with 1 ml of 75% ethanol by vortexing and centrifuging at a maximum of 7500 RCF/G for 5 min at 2 to 8°C. The supernatant was removed and the RNA pellet was dried and dissolved by pipetting in 40µl DEPC-treated water. DNase digestion was made by adding 40 U of recombinant RNasin Ribonuclease inhibitor (Promega, Mannheim), 10µl 5x first strand buffer (Invitrogen, CA, USA), and 2µl RQ1 RNase –Free DNase (Promega, Mannheim) to the RNA and incubated at 37°C for 1 hr in an air incubator (Heraeus). Each sample of isolated total RNA was tested by electrophoresis, loading 2µl total RNA mixed with 2µl loading buffer (Biomol). The mix was run at 125 V for 15 min by using a 1.2% FA agarose gel (1.2g agarose, 100µl 10x FA buffer, 40 mg EtBr) submerged in an electrophoresis pool (BioRad, Munich) containing 1X FA gel running buffer (10 X FA buffer, 37% formaldehyde and DEPC-treated water). Two bands were seen through an Uvi-Tec Transilluminator and analysed by the gel doc system (Uni-Equip, Martinsried).

Because the total RNA includes residual RNAs and RNases, the RNeasy MiniElute™ Protocol (Qiagen, Hilde) was employed for a clean-up following the handbook instructions. At first, in a 2 ml Mµlti®-Reaktionsgefäße tube (DNase/RNase-frei, Carl Roth GmbH), samples were adjusted to a volume of 100µl with RNase- free water (Qiagen, Hilde). 350µl of RLT buffer (containing guanidine thiocyanate and ß-mercaptoethanol) was added and mixed thoroughly by shaking to clean out RNases, immediately 250µl of 100% ethanol was added and mixed very well by pipetting. The 700µl of the mixed sample was transferred to RNeasy mini-spin column assembled to a new 2 ml Mµlti®-Reaktionsgefäße tube (DNase/RNase-frei, Carl Roth GmbH) for the flow-through collection and centrifuged at a maximum of 8 000 RCF/G (10 000 rpm) for 15 sec.

The RNeasy spin column was transferred into a new 2 ml Mµlti®-Reaktionsgefäße tube (DNase/RNase-frei, Carl Roth GmbH) for collection and 500µl RPE buffer was added and centrifuged for 15 sec at 10 000 rpm to wash the RNA incrusted in the RNeasy spin column membrane. At that moment 500µl RPE buffer was pipetted into the same RNeasy column, and centrifuged for 2 min at maximum speed to dry the RNeasy spin column membrane. Once the RNeasy column was transferred into a new 2 ml microcentrifuge tube, 30µl of RNase free water was pipetted directly into the RNeasy membrane and centrifuged for 1 min at 10 000 rpm to elute the cleaned RNA. The RNeasy membrane was again washed with 20µl RNase free water to recover as much cleaned RNA as possible. To test RNA quality, 2µl of total RNA was mixed with loading buffer (2 µl) and loaded in a 1.2% FA gel to be electrophoresed as explained above.

Concentration was determined in 1ml UV polyestrol cuvette (Plastibrand®-Labomedic) by means of absorbance through an UV - visible light DU-62 series spectrophotometer (Beckman, Munich, Germany) to obtain both A_{260} and A_{280} ratios, using the formula RNA (µg/µl) = [OD_{260} X 40 X DF] / 1000. 496µl 1XTE buffer plus 4µl of diluted DNA (1:125) was used. Aliquots were prepared to achieve 0.50 µg/µl to work in a reverse transcription PCR reaction. The aliquots of isolated total RNA were examined by electrophoresis using a 1.2% FA agarose gel as previously explained.

3.2.5 cDNA synthesis

3.2.5.1 cDNA synthesis by SuperScript II RNase reverse transcriptase

Before cDNA synthesis, each RNA sample was tested in a PCR using primers for a housekeeping gene to test gDNA contamination and fidelity of the procedure. After that, when the RNA was DNA free, it was reverse transcribed to the single strand complementary DNA synthesis by means of SuperScript II RNase reverse transcriptase (Invitrogen, Karlsruhe, Germany). Oligo-T7 primer or GS primers were used to obtain a 20µl volume reaction. At first, 1µl of T7 oligo (20µM) or GS primers (10 pmol/µl) were mixed with 2µg of total RNA isolated by means of Trizol® reagent (Gibco BRL, Life Technologies) system or 11µl of mRNA isolated by means of Dynabeads Oligo dT 25 (Dynal Biotech ASA, Oslo, Norway) system in a new 0.2 ml PCR tube (PCR-0208-C,

Axygen®) and incubated at 70°C for 10 min in a PTC-100^{TM} programmable thermal controller (MJ Research, Inc.). The mix was then chilled for 2 min on ice block. At the end, master mix containing 4µl of 5X first strand buffer (50mM Tris-HCL, pH8.3; 75mM KCL; 3mM $MgCl_2$), 2µl DTT - (0.1 M) and 10 mM dNTP mix (containing 100 mM dATP, 100 mM dCTP, 100 mM dGTP and 100 mM dTTP), 1µl (200 U) of Superscript II RT (Invitrogen, Karlsruhe, Germany) was prepared and added to the RNA mix. Immediately, the solution was mixed gently by pipetting and incubated at 42°C for 1 hr and at 70°C for 5 min, at the end of thermo-protocol the samples were chilled on ice bath.

3.2.5.2 cDNA synthesis by RACE first-strand

In order to amplify the 3' and the 5' tails of a gene from a EST, RACE ready first-strand cDNA was constructed by using the BD SMART^{TM} RACE amplification kit (BD Biosciences Clontech). It was used as a template in the named RACE-PCR reaction for both the sense and the antisense strands. In this technique, a pooled postimplantative total RNA solution (0.5 µg/µl) was used, which was added in two separated 0.2 ml PCR-0208-C, Axygen® tubes.

To construct the 5'-RACE-ready cDNA was mixed with 1 µg total RNA, 1µl 5'-CDS primer (BD Biosciences Clontech), 1µl SMART II A oligo (BD Biosciences Clontech), and DEPC H_2O to fill at 5µl. For the 3'-RACE-ready cDNA 1 µg total RNA, 1µl 3'-CDS primer A (BD Biosciences Clontech) and DEPC H_2O were mixed to get 5µl. Each mix was pipetted very well and incubated in a PTC-100^{TM} programmable thermal controller (MJ Research, Inc.), with a thermal protocol of 70°C for 2 min and cooling at 0°C for 2 min.

After that to each 0.2 ml PCR-0208-C, Axygen® tube, which already contains 5µl of the first mix, 2µl 5X First-Strand buffer, 1µl DTT (20 mM), 1µl dNTP Mix (10 mM), 1µl BD Power Script reverse transcriptase were added to a 10µl total volume. The reaction was mixed by gently pipetting and centrifuged briefly. Moreover, samples were incubated at 42°C for 1.5 hr and at the end 100µl of Tricine-EDTA buffer were added to dilute the first-strand reaction product that was heated at 72°C for 7 min. The 3'- and 5'-RACE-ready cDNA samples were eventually stored at –20°C to be used as a template for the following RACE-PCR reaction.

3.2.6 Identification of homeobox genes EST´s by PCR

3.2.6.1 Polymerase chain reaction

The isolated material and the provided commercial reagents and media as well as the designed GS primers were used as row materials for PCR reactions as explained below. All isolated gDNAs and RNA as well as the synthesized cDNA were tested by using 2µl of each sample, for functionality or gDNA contamination respectively. A Housekeeping gene (bovine ß-actin) was used for this purpose. It amplifies a 263 nt amplicon from bovine complementary DNA (mRNA) and a 370 nt amplicon in bovine gDNA to look for differences (107 nt) in an agarose gel, the rest of the solution was stored at –20°C for subsequent works.

Using a 0.65 ml microcentrifuge tube (Carl Roth GmbH-Mµlti®-Reaktionsgefäße/DNase/RNase-frei) a reaction was prepared, which contains 0.1µl (0.5U) of Taq DNA polymerase (Sigma-Aldrich-GmbH), 1X PCR buffer (Sigma-Aldrich-GmbH, 10 mM Trisma®-HCl, pH 8.3 at 25°C, 50 mM KCl, 1.5 mM $MgCl_2$ and 0.001% (w/v) gelatine), 0.5 µM of each sense or antisense degenerated primer, 10mM of dNTP mix, ddH_2O. 2µl cDNA pooled from postimplantative embryos was also used to make PCR.

The objective of this PCR was to sequence homologous fragments of different Hox-gene members by subcloning of the derived bands containing a heterogeneous group of 166 nt amplicons and to sequence a random of selected clones containing each amplicon (Adjaye and Monk 2000, Ponsuksili et al. 2001a, b).

To find the 3' and the 5' tails of Cdx1 and Cdx2 ESTs through the BD SMART^{TM} RACE system (BD Biosciences Clontech), two RACE-PCR reactions were performed for each Cdx1 and Cdx2 gene following the kit manual instructions. A 0.65 ml microcentrifuge tube (Carl Roth GmbH-Mµlti®-Reaktionsgefäße/DNase/RNase-frei) was used for each. The reaction mix contains 3µl of 3'- or 5'-RACE-Ready cDNA, 2µl 10X BD Advantage 2 PCR buffer, 0.70µl dNTP mix (10nM), 0.20µl BD Advantage 2 Polymerase mix, 0.50µl UPM 10X primer, 0.50µl 3' or 5' GSP (10µM) respectively, and 13.10µl DEPC H_2O. The reaction mix was run for an initial denaturation at 94°C for 3 min and 40 cycles of 94°C for 30 sec denaturation, 68°C for 30 sec annealing, 72°C for 3 min extension and a final extension at 72°C for 3 min.

A second round of RACE-PCRs were run in a touch down thermal cycler protocol with an initial denaturation at 94°C for 5 min, 10 cycles of 94°C for 15 sec, 70°C for 15 sec (-0.5°C each cycle) annealing, 72°C for 3 min extension, 94°C for 15 sec and 35 cycles of 65°C 15 sec for annealing, 72°C for 3 min for extension, and a final extension at 72°C for 10 min. For both RACE-PCRs, products were run in 0.7% agarose gel containing 5µl EtBr (Roth, Karlsruhe) (10mg/ml) at 125 V for 40 min.

The third way was designed to work with anchored-primers, as it was explained in the primer design, to amplify a desired fragment from a gene. PCR conditions were used as explained below and run in a touch down PCR analogous as explained above, and run in a PTC-100^{TM} programmable thermal controller (MJ Research inc.).

The fourth system followed was to find ESTs of primers established by multiple sequence alignment. Because, the Tm of the primers was unknown, the PCRs protocols were established in a combined step and touch down PCR that run in a PTC-100 ^{TM} programmable thermal controller (MJ Research inc.).

The thermal protocol consisted of 5 min at 95°C as an initial denaturation step, after that 2 cycles of 15 sec at 95°C, 1 min at 70°C and 1 min at 72°C, followed by a step of 14 cycles at 95°C for 15 sec, 69°C for 15 sec and 72°C for 1 min, -1 °C each cycle, then 21 cycles of 95°C for 15 sec, 55°C for 15 sec and 72°C for 1 min, and a final extension step of 3 min at 72°C.

The master mix for each pair of primers to be analysed consisted of 0.1µl (0.5U) of Taq DNA polymerase (Sigma-Aldrich-GmbH), 1X PCR buffer (Sigma-Aldrich, 10 mM Trisma®-HCl, pH 8.3 at 25°C, 50 mM KCl, 1.5 mM $MgCl_2$ and 0.001% (w/v) gelatine), 0.5 µM of each forward or reverse primer, 10mM of dNTP mix, 2µl cDNA or gDNA as template and ddH_2O. PCR products were loaded as previously explained and run at 50 V for 60 min in a 1% agarose gel cooked with 4µl EtBr (Roth, Karlsruhe) (10mg/ml) for each 100 ml 10X TAE buffer and run together with 5µl of λ Eco 91I marker.

To establish the optimal Tm of designed primers gradient PCR protocol was used. This method helps us to make a PCR looking for the best annealing temperature for each par of primers in eighth reaction protocols. Thus, the PCR was run in a BioRad I cycler thermo controller (BioRad), where the master mix for each pair of primers was composed of the established 20µl reaction as explained above.

The thermal protocol consisted of an initial denaturation step of 5 min at 94°C, and then 35 cycles of 30 sec for denaturation at 94°C, 30 sec for annealing at 50 to 60 or 58 to 70°C, 1 min for extension at 72°C and a final extension step for 5 min at 72°C. 15µl PCR products plus 3µl loading buffer ran at 125 V for 25 min in a 1% agarose gel and 4µl EtBr (10 mg/ml-Roth, Karlsruhe) for each 100 ml 10X TAE buffer.
After that, as all primers took annealing at 60°C without large differences. Following the recommendations of the ABI PRISM® system, a PCR protocol was established to know whether primers could work at 60°C with high efficiency. A thermal protocol was standardized at an initial denaturation at 94°C for 5 min, 35 cycles of denaturation 94°C for 30 sec, 60°C for 30 sec for annealing, 72°C for 1 min for extension, and a final extension step of 72°C for 5 min. The 5µl of PCR products plus 1µl of loading buffer ran in a 2% agarose gel and 4µl EtBr (Roth, Karlsruhe) (10 mg/ml) for each 100 ml 10X TAE buffer, at 125 V for 20 min. 2µl of lambda DNA/Bst EII marker (500ng/µl) ran together.
All PCR products were analysed by means of a UV Transilluminator (Uvi-tec) and the gel doc system (Uni-Equip, Martinsried, UK). All handling of equipment, products and reactions containing EtBr (Roth, Karlsruhe) were operated using globes.

3.2.7 Isolation of PCR products from agarose gels

The electrophoresed PCR products were excised from the agarose gel with a new Cutfix® surgical disposable scalpel (B-Braun-Aesculap, Tuttlingen) and put in a new 2 ml Mµlti®-Reaktionsgefäße tube (DNase/RNase-frei, Carl Roth GmbH) to be purified by the phenol - chloroform method analogous as explained above (Sambrook et al. 1989). The excision was done direct on the UV transilluminator (Uvi-tec) using a faceshield 3C - headbread (Fibre Matal®, USA). 500µl of 1X TE were added to the agarose gel bit.
The tube was previously warmed at a maximum of 25°C to facilitate the gel bit to be completely cracked. With an 18 gauge (1.27mm) needle, small gel fragments were totally homogenized by inflow and outflow using a 10ml syringe with needle (20 gauge - 0.902mm). 500µl phenol-chloroform (1/1 v) were added and homogenized in the same form and then the mix was centrifuged for 20 min at 10 000 rpm. After that the upper phase of the sample was transferred into a new 2 ml Mµlti®-Reaktionsgefäße tube

(DNase/RNase-frei, Carl Roth GmbH). 500µl chloroform (1 v) was added, the tube was vigorously shaken and centrifuged for 20 min at 10 000 rpm.
Afterwards the upper phase was transferred to a new 2 ml Mµlti®-Reaktionsgefäße tube (DNase/RNase-frei, Carl Roth GmbH). 50µl 3M sodium - acetate (1/10 v) was added to precipitate the DNA amplicon, and double volume of 100% ethanol was added, vigorously shaken and incubated for 2 hr at –80°C or overnight at -20°C. The product was centrifuged for 1 hr at 10 000 rpm, the fluid phase was discarded and the pellet was washed two times by adding 50µl of 5% ethanol and vortexing. The mix was centrifuged at 10 000 rpm at 4°C for 30 min. The fluid phase was removed and the pellet was dried at air for 20 min and eventually it was dissolved by pipetting 10µl sterilized Millipore® Milli Q water and stored at 4°C or at -20°C for further use. 1µl of isolated product plus 2µl loading buffer was loaded in 1% agarose gel and ran by electrophoresis at 125 V to confirm the result by using the gel doc system (Uni-Equip, Martinsried, UK).

3.2.8 Ligation of the PCR amplicon

To achieve the PCR amplicon analysis by cloning and sequencing, the pGEM® -T easy vector system ligation kit (Promega) was employed. The pre-mix, containing 2.5µl of 2X Rapid ligation Buffer T4 DNA Ligase, 0.5µl of pGEM® -T vector (50ng/µl) and 0.5µl T4 DNA Ligase (3 U/µl), was mixed by pipetting with 1.5µl of the isolated PCR product in a 0.2 ml Thin-wall PCR 0208-C tube with cap (Axigen Scientific, USA). A 3.5µl reaction was incubated for 1hr at room temperature (20 to 25°C), 2hr at 15° using a PTC-100 ™ programmable thermal controller (MJ Research, Inc.) or overnight at 4°C in a refrigerator. When the PCR product was not sharp and bright at the agarose gel doc system (Uni-Equip, Martinsried, UK) analysis, a direct ligation of 1.5µl PCR product was performed without PCR clean up to avoid loss of product.

3.2.9 Transformation of ligated fragments

Competent cells used for transformation were transported in ice bath from the -80°C freezer to be thawed. Thus 60µl of competent cells were added to a 15 ml falcon PP-Test sterile tube (Cellstar) containing 3µl of ligation product for each sample,

homogenized by gently shacking with the finger and incubated for 30 min in ice bath. Subsequently, samples were incubated in a water bath at 42°C exactly for 90 sec without shaking, by the known method of heat-shock (Sambrook et al. 1989), to permit the introduction of the pGEM® - T vectors (Promega) with or without amplicon into the *E. coli* competent cells. Soon after the heat shock, each sample was placed in ice bath for 2 min to permit the closing of the *E. coli* competent cells. 700µl LB-broth without ampicillin was added to the 15 ml falcon PP-Test sterile tube (Cellstar) placed at 60° of inclination and incubated at 37°C for 80 min at 100 rpm in a shaking incubator (Thermoshake Gerhardt, John Morris scientific) to allow the development of *E. coli* competent cells with or without the pGEM®-T vector.

In a laminar flow cabinet Hera-safe (Heraeus) was prepared one Pasteur pipette for each sample, formerly polished by flame at the tip with a gas-burner and shaped in "L" form. Also, two Petri dishes as duplicated were prepared by adding warm LB-broth agar with ampicillin (25µl ampicillin (10 mg/ml)/50ml LB-broth-Agar) for each sample. After cooling, 20µl X-Gal-dimetilformamide (50mg/ml working solon) and 20µl isopropylthio-ß-D-galactoside (IPTG-0.5 M-40µg/µl) were added and spread over the surface by using the "L" form Pasteur pipette.

380µl of the transformed competent cells was poured on the surface of each Petri dish, and incubated at 37°C in an air incubator (Heraeus) overnight, approximately for 16 hr. Colonies were identified through the amplicon insertion and inactivation of the α-peptide in the coding region of the ß-galactosidase gene that permit the color screening identification.

After colony identification (approximately 1mm diameter) in a laminar flow cabinet (Nunc GmbH & Co. KG), for each Petri dish, 2 white colonies (with the cloned amplicon) and 1 cobalt-blue colony (without cloned amplicon as control) were picked up using fire-sterilized loop. A M13 - PCR with primers taking annealing in the pUC/M13 fraction of the pGEM®-T vector (Promega) ran to check the correct amplicon transformation.

Three Thin-wall PCR 0208-C tubes with cap (Axigen Scientific, USA) for each Petri dish were previously prepared by adding 30µl of 1 X PCR buffer (1µl 10X PCR buffer for each 9µl Millipore® Milli Q H_2O).

The loop containing clones was very well mixed in the buffer by spinning. After the mixing of each positive sample, the loop containing colonies dilution was introduced in

a 2 ml Mµlti®-Reaktionsgefäße tube (DNase/RNase-frei, Carl Roth GmbH). The tube contained 1.5 ml LB-broth-ampicillin (1µl ampicillin (10 mg/ml), for each 2 ml LB-broth) that was incubated at 37°C overnight (24 hr) in the shaking incubator (Thermoshake Gerhardt, John Morris scientific) at 108 rpm to allow the growth of *E. coli* competent cells with the pGEM® -T vector and the PCR amplicon. Clones mixed with the 30µl of 1 X PCR buffer were boiled at 95°C for 15 min and used as soon as possible in the next step.

3.2.9.1 M13 PCR protocol to identify cloned amplicons after transformation

After each transformation procedure M13-PCR protocol was performed to check the cloning success and take the decision to sequence. The pUC/M13 fragment contained 219 or 371 nt of the pGEM® - T vector (Promega) plus the PCR amplicon. Forward - TGTAAAACGAACGGCCAGT and reverse - CACCAAACAGCTATGACC were used to amplify 219 bp plus insert and forward-GCTATTACGCCAGCTGGCGAAAGG and reverse-CCCCAGGCTTTACACTTTATGCTTCC that amplify 371 nt plus insert. There are different possibilities to work with the plasmid DNA (pDNA) or recombinant plasmid DNA (recDNA) (Sambrook et al. 1989).

In a 0.65 ml microcentrifuge tube (Carl Roth GmbH-Mµlti®-Reaktionsgefäße/DNase/RNase-frei) a mixture of 0.1µl (0.5U) of Taq DNA polymerase (Sigma-Aldrich-GmbH), 1X PCR buffer (Sigma-Aldrich, 10 mM Trisma®-HCl, pH 8.3 at 25°C, 50 mM KCl, 1.5 mM $MgCl_2$ and 0.001% (w/v) gelatine), 0.5 µM of each forward or reverse primer, 10mM of dNTP mix, and ddH_2O was prepared. 10µl boiled colonies solution was used to complete a 20µl PCR reaction.

The thermal protocol consisted of an initial denaturation at 94°C for 5 min, 35 cycles of 94°C for 30 sec, 59 or 65°C for 30 sec step according to the M13 mixed primers, 72°C for 1 min, and a final extension step of 72°C for 5 min. The protocol was used in a PTC-100^{TM} programmable thermal controller (MJ Research, Inc.). 5µl of PCR product plus 1µl of loading buffer ran in a 2% agarose gel containing 4µl EtBr (10 mg/ml-Roth, Karlsruhe) for each 100 ml 10X TAE buffer, at 125 V for 15 to 25 min, 1µl of lambda DNA/Bst EII marker (500ng/µl).

3.2.10 Sequencing of PCR amplicons by Sequiterm Exel II protocol

Sequencing of ESTs, as well as the confirmation of gene-specie-specific amplicons to accomplish the real-time PCR quantification, were achieved by employing a system based on the Sanger method of dideoxid mediated chain termination (Sambrook et al. 1989). The SequiTherm EXEL™ II DNA system (Epicentre® - Madison, WI, USA) making use of the LI-COR DNA scanning system (LI-COR, Inc., Biotechnology division, Lincoln, Nebraska, USA).

The SequiTherm EXEL™ II DNA system (Epicentre®, Madison, WI, USA) was used following the manufacturers instructions. Samples were always manipulated on ice block. 2.5µl of selected M13 - PCR product or anchored - PCR product, 5.40µl of 3.5 X SequiTherm EXEL II sequencing buffer, 0.75µl of SequiTherm EXEL II DNA Polymerase (5 U/µl), 0.30µl of each 5´ IRD 800 T7 and 5´ IRD 700 SP6 labelled primers (2.5 pmol), and ddH_2O were mixed into a 0.65 ml microcentrifuge tube (Carl Roth GmbH-Mµlti®-Reaktionsgefäße/DNase/RNase-frei).

For each sample sequenced, 2.25µl of above mix was added into each Thin-wall PCR 0208-C tube (Axigen Scientific, USA) containing 1µl of SequiTherm EXEL II termination Mix A, C, T or G. Samples were mixed very well and quickly centrifuged and ran in a PTC-100™ programmable thermal controller (MJ Research, Inc.) at 94°C for 3 min, 29 cycles of 94°C for 15 sec, 54°C for 15 sec, 69°C for 1.20 min and a final chilling at 4°C.

At the end of the thermal protocol, 1.5µl of stop-loading buffer was added and denatured at 95°C for 5 min in the same thermal controller or stored at -20°C. 1 to 1.5µl of sample was loaded in a 66 cm long boroflat glass sandwich previously prepared in the rail-assembly apparatus for electrophoresis. A 48-well sharks tooth comb was inserted in the SequaGel® (National diagnostics, USA) sequencing gel. Reactions were run overnight by using LI-COR 4200 series DNA scanning system (LI-COR, Inc., Biotechnology division, Lincoln, Nebraska, USA).

Image was saved and analysed by the Base ImageIR™ reference manual version 4 software (LI-COR, Inc., Biotechnology division, Lincoln, Nebraska, USA). The 20 mer 5´ IRD 800 T7 labelled primer 5'-d-TAATACGACTCACTATAGGG-3' and the 24 mer 5´ IRD 700 SP6 labelled primer 5'-d-CATACGATTTAGGTGACACTATAG-3' (Epicentre®, Madison, WI, USA) primer sequences were used.

3.2.11 Plasmid DNA isolation to perform serial dilutions

After the sequence analysis, *E. coli* competent cells containing the PCR amplicon were selected as target template. recDNA-GS was isolated by means of the GeneElute™ Plasmid Miniprep Kit (Sigma-Aldrich-GmbH) direct from the 1.5 ml LB-broth-ampicillin containing *E. coli* competent cells including the PCR amplicon after 24 to 32 hr of incubation. If the 1.5 ml LB-broth-ampicillin contained a few amount of *E. coli* competent cells after the overnight by shaking incubation, the whole amount was transferred to a new 15 ml falcon PP-test sterile tube (Cellstar) containing 10 ml LB-broth plus 5µl ampicillin (10 mg/ml). It was done in a previously sterilized laminar flow cabinet (Nunc GmbH & Co. KG) and incubated in the shaking incubator (Thermoshake Gerhardt, John Morris scientific) at 37 °C overnight at 108 rpm.

The recombinant *E. coli* culture containing amplicon was compacted by centrifugation at 12,000 RCF/G for 1 min at room temperature. The fluid phase was discarded and the pellet was resuspended by pipetting with 200µl of the resuspension solution containing RNase A solution. Recombinant *E. coli* strains were lysed by adding 200µl of the lysis solution and mixed by moderate inversion until the mixture becomes clear and viscous. The solution was neutralized and the cell debris was precipitated by adding 350µl of neutralization/binding solution and gently shaking. The cell debris was pelletized by spinning at 12,000 RCF/G for 10 min. Previously, a GenElute® Miniprep binding column was inserted into a provided 2ml collection tube and humidified with 500µl of the column preparation solution and centrifuged at 12,000 RCF/G for 1 min.

The upper phase as a cleared lysate was transferred to the prepared GenElute® Miniprep binding column and centrifuged at 12,000 RCF/G for 1 min, and the flow-through liquid was discarded. The column was washed with 750µl of 100% ethanol/wash solution and centrifuged at 12,000 RCF/G for 1 min. Washing was performed two times. The flow-through liquid was discarded and the GenElute® Miniprep binding column centrifuged again at maximum speed for 1 to 2 min without any additional wash solution to remove excess ethanol.

The column was transferred to a new collection tube to elute the recDNA-GS by adding 100µl of elution solution, Millipore® Milli Q water or 5 mM Tris-HCl, pH 8.0 as an eluant, and centrifuged at 12,000 RCF/G for 1 min. The recDNA-GS size and quality was determined by electrophoresis in a 2% agarose gel containing 4µl EtBr (10 mg/ml-

Roth, Karlsruhe) for each 100 ml 10X TAE buffer, ran in a BioRad® electrophoresis pool (BioRad®) at 125 V for 30 min and compared with 5µl marker (λ Este II Marker). 10µl recDNA-GS solution was transferred and vortexed in a new 2 ml Mµlti®-Reaktionsgefäße tube (DNase/RNase-frei, Carl Roth GmbH) containing 390µl Millipore® Milli Q water to measure concentration. A 1µl UV polyestrol cuvette (Plastibrand®-Labomedic) and a UV-visible light DU-62 series spectrophotometer (Beckman, Munich, Germany) in 260 and 280 of optical density were employed.
The ratio of absorbance at 260 nm to 280 nm (A_{260}/A_{280}) was between 1.7 and 1.9, as DNA in ng/µl was determined by the formula [OD_{260} X 50 X DF] = [OD_{260} X 50 X 40], where DF is the dilution factor (v total of Solution / v of DNA solution) at 40 and OD_{260}= optical density in 260nm.

3.2.12 Gene expression by real-time PCR system

Following the relative standard curve method, the quantitative real-time gene expression analysis for each EST was performed using an ABI PRISM® 7000 sequence detection systems thermo controller. SYBR ® Green (Sigma-Aldrich-GmbH) was used as a double-strand DNA-specific fluorescent dye. Analyses were made using the ABI PRISM® 7000 SDS- CTS 1.0 software (Sigma-Aldrich-GmbH), and results were analysed by Microsoft EXCEL (Bustin 2000, 2002, Robert et al. 2002, Garcia-Castillo et al. 2002). Quantification compares the point of dye fluorescence of an established standard curve with known molecules copy number against the unknown sample.
To prepare an absolute standard curve, the double stranded DNA amplicon standard method was applied (Bustin 2000, Bustin et al. 2005). The method involves subcloning of the PCR amplicon within the T7-SP6 RNA polymerase promoters of the pGEM® -T (Promega) plasmid vector. The calibrator can be any nucleic acid chain; as long as its concentration and length of amplicon are known, specific amplicons of known genes were chosen. The target C_T was compared directly with the calibrator C_T and was recorded as containing either high or low amplicon, which is relative to the mRNA. For such reason the procedure explained below was carried out.

3.2.12.1 Preparation of aliquots from plasmid DNA (recDNA-GS) solution for real-time PCR standardization

User Bulletin #2 by ABI PRISM® (http://docs.appliedbiosystems.com/pebiodocs/04303859.pdf), was followed in order to perform the absolute standard curve for the real-time quantification of gene expression (Pfaffl 2001, 2003). Aliquots in serial dilution were prepared with the isolated recDNA-GS solutions. This was done by conversion of the fragment size and its weight from the spectrophotometry. The quantity of amplicon molecules per µl of recDNA-GS solution was estimated with the help of the following formulas:

$$\text{Molecules of amplicon DNA} = Q \times N_A$$

$$Q = m / Mw \times L \times 10^3$$

or

$$Q[\text{pmol}] = m[\text{pg}] / L[\text{b}] \times Mw\,[\text{Da}] = m[\text{pg}] / L[\text{b}] \times 649\ \text{Da}$$

$$Q[\text{mol}] = c[\text{M}] \times V[\text{L}]$$

$$m[\text{g}] = Q[\text{mol}] \times Mw[\text{Da}] \times L[\text{kb}] \times 10^3$$

Molecules of amplicon DNA = quantity of molecules of nucleic acids in the amplicon.

Q = quantity of nucleic acid (in the same tube) in moles.

N_A = Avogadro constant = 6.022045×10^{23}[1/mole].

m = weight of nucleic acid in grams.

Mw = mean molecular weight of one or a pair of nucleotides in Daltons (base= 324.5Da, base pair = 649Da, ribo base = 340.5Da).

L = length of nucleic acid chain in thousands of bases (Kb) containing recDNA-GS plus amplicon.

c = molar concentration of nucleic acid in Mole (M = mole/litre).

V = dilution volume per litre.

To get the 10^{10} or 10^9 molecules per µl of the desired amplicon containing recDNA-GS as an initial point in the standard curve was done by means of the formula:

$$M_1 \times V_1 = M_2 \times V_2$$

Where:

V_1 = required volume from the original solution in µl.

M_1 = amount of molecules in the original solution.

V_2 = volume total in the aliquot to be prepared in µl.

M_2 = desired molecules amount in the wanted aliquot.

$$V_1\mu l = (M_2 \text{ molecules X } V_2\ \mu l) / M_1 \text{ molecules}$$

With these systems 50µl aliquots of recDNA-GS solution containing 10^{10} molecules of amplicon- recDNA-GS were eventually obtained. Therefore from such aliquot were pipetted 5µl of recDNA-GS plus 45µl water to fill a 50µl aliquot with 10^9 molecules of amplicon- recDNA-GS for each µl, and so on this repeatedly form serial dilutions were worked until obtain an aliquot of 10^1 molecules of amplicon- recDNA-GS for each µl.

With these was established the standard curve for each Hox-gene to be quantified and for the nucleosomal housekeeping gene Histon-2A (H2AFZ, X52318), that work in the chromatin structure. Employing endogenous quantitative reference genes is finer as make use of exogenous in vitro references, but some reference genes are superior to others (Radonic et al. 2004). Therefore here was selected H2AFZ gene to the results normalizing as an endogenous standard, since its mRNA can be used as a stable quantitative marker (Lonergan et al. 2003b, McGraw et al. 2003, Fair et al. 2004a, b, Pfaffl et al. 2004). H2AFZ has been the best option to be employed in the analysis of mRNA expression in bovine preimplantation embryos, because its constant expression across this period (Robert et al. 2002).

After this step a standardized PCR protocol for each serial dilution of recDNA-GS containing specific-amplicon was run to check amplification, serialized concentration and to detect primer dimers or secondary structures. For this were mixed 0.5 µM of each corresponding GSS primers (10 pmol/µl), 0.1µl (0.5U) of Taq DNA polymerase (Sigma-Aldrich-GmbH), 1 X PCR buffer (Sigma-Aldrich, 10 mM Trisma®-HCl, pH

8.3 at 25°C, 50 mM KCl, 1.5 mM $MgCl_2$ and 0.001% (w/v) gelatine), 10mM of dNTP mix, 2µl of recDNA-GS serial dilutions as template and ddH_2O. The thermal protocol consisted of 95°C for 5 min, 35 cycles of 95°C for 30 sec, 60°C for 30 sec, 72°C for 1 min and a final extension of 72°C for 5 min. PCR products were loaded in a 1% agarose gel with 4µl EtBr (10mg/ml-Roth, Karlsruhe) for each 100 ml 10 X TAE buffer and ran at 125 V for 15 to 20 min, and analysed as explained above.

3.2.12.2 Optimization of GSS primer concentration for real-time PCR

The concentration of each designed pair of GSS primers for the real-time quantitative PCR was optimized according to the recommendations of Applied biosystems. For this PCR, three levels of each sense and antisense GSS primers (50, 300 and 900 nM) were employed in the standardized PCR protocol for recDNA-GS serial dilutions, resulting in nine different combinations for each one pair. In this regard, 2µl of the recDNA-GS aliquot which showed the smaller amount of primer dimer in the recDNA-GS serial dilution PCR were used as template. Each combination was run against 2µl Millipore® Milli Q water as negative control for each primer combination, identified in the system as the no template as control (NTC). This PCR was carried out in an ABI PRISM® 7000 sequence detection systems thermo controller (Applied biosystems, Foster city, CA, USA), where the optimal primer combination was selected.

Using each optimal primer combination was carried out a PCR protocol for each gene to be analysed, but using recDNA-GS serial dilutions on the same form as explained above. For this serial dilutions PCR was employed the ABI PRISM® 7000 sequence detection systems thermo controller (Applied biosystems, Foster city, CA, USA). The aim was to obtain a standard curve performed automatically by the ABI PRISM® 7000 SDS-CTS 1.0 software (Applied biosystems, Foster city, CA, USA). Hence, recDNA-GS serial dilutions were fined until obtain the optimal PCR amplification with the best possible concentration of GSS primers combination avoiding nonspecific amplification. At this moment was searched the lowest C_T in quest of an optimal melting curve analysis.

3.2.12.3 mRNA expression quantification by real-time PCR

The two steps real-time semi quantitative PCR was performed using pooled bovine embryo samples as explained above. For this, the rest of the 20µl mixed reaction was composed of 0.20µl of Internal Reference (Sigma-Aldrich-GmbH), volume optimized of primers from a dilution of 10 pmol/µl, ddH_2O, and 2µl of recDNA-GS serial dilutions as template, or 2µl of bovine embryo cDNA for quantification. Mixed solutions have been worked always on ice block and vortexed in a 1,7 ml black-colour micro centrifuge tube (Carl Carl Roth GmbH-Mµlti®-Reaktionsgefäße/DNase/RNase-free) as master mix, that was added (18µl) in an iCycler iQ™ PCR Polypropylene 96 wells plate (Bio Rad - DNase/RNase-free) previously containing the template.
The automated thermal profile for all optimization PCRs as well as for quantification PCRs consisted of a first stage of 50°C for 2 min to activate 10µl SYBR® Green JumpStart™ Taq Ready Mix™ (Sigma-Aldrich-GmbH), a second stage of 95°C for 10 min, a last stage of 40 repetitions of 95°C for 51 sec and 60°C for 1 min. At the moment of the real-time PCR in each repetition of the last stage, during the ramping time from 60°C to 95 °C, laser light measurements were automatically took every 7 sec that look for the starting of the fluorescence emission. These measurements generated the dissociation curve for each reaction, which was employed to test the specificity of PCR and the presence of primer dimer formation and for calibration.
Thus expression quantification from the two-step real-time PCR has been shown in terms of relative changes in the C_T value. This is referred to the PCR cycle number or point in time where the amplification of the target cDNA or gene fragment has been detected at first time throughout the fluorescence intensity reflected from the attached binding SYBR green II as dye, and the time when it is greater than the background fluorescence. It is supposed to be the exponential amplification where such PCR amplification crosses the set C_T from cycle threshold, also identified as crossing point (CP) (Larionov et al. 2005, Wong and Medrano 2005).

The threshold is defined as:

$$\Delta R_n = (R_n^+)-(R_n^-)$$

R_n = magnitude of the signal generated by the given set of PCR conditions.

R_n^+ = distribution of the emission intensity of the SYBR Green among the emission intensity of the passive reference in the PCR containing a template.

R_n^- = distribution of the emission intensity of the SYBR Green among the emission intensity of the passive reference in the PCR without template or at early cycles of a real-time PCR.

C_T value indicates the point where the fluorescence spectrum increase statistically significant in the magnitude of the signal generated. This was done by distributing a laser light via a multiplexed array of optical fibres directed to a spectrograph with a charge-coupled device (CCD) camera. The camera begins to detect the signal increasing associated with an exponential growth of PCR product to be recorded as the average standard deviation from the early cycles and multiplied by an adjustable factor.

The amplicon copies number was automatically calculated after the real-time PCR from the linear regression of an absolute standard curve. For this, the absolute standard curve has been performed from the C_T value of each recDNA-GS serial dilution worked. The smaller value of C_T is the higher concentration and the bigger C_T value is equivalent to the lower concentration in the serial dilutions. Thus C_T value is inversely proportional to the log of the initial copy number (Higuchi et al. 1993, Bustin et al. 2000, 2005). For that reason by calculating the C_T values against the logarithm of the initial amplicon copy number can be generated such absolute standard curve, with a confidence interval more than 95%.

The quantity for samples was calculated by this method as an ordinary linear function formed from the C_T PCR result of the serial dilution of the concentration at 10^9 molecules of recDNA-GS to give a final concentrations of 10^2 recDNA-GS copies by trying to achieve a coefficient of determination around 99% and resulting of the elevation of 10 to the result of the subtraction of the intercept from the C_T value divided by the slope.

$$10^{([\text{CT value- intercept-}]/\text{ slope})}$$

PCR amplification efficiencies and exponential amplification, both were calculated by using the equations (see below), to be ideal the PCR efficiency around the 1, this corresponding to a supposed slope of -3,322009 from the linear regression. Results of each PCR have been normalized by analysing the H2AFZ (X52318) gene expression, in the same sample analysed for each Hox gene, by both methods C_T and quantity. These since the relation between C_T values and RNA possible fragmentation, which by the calculation of delta C_T value could compensate the degradation-related shifts over such C_T values took from the PCR. Delta C_T values are unaffected by changing amounts from the analysed mRNA, even by their fragmentation (Antonov et al. 2005, Bustin 2000, Wong and Medrano 2005).

$$\text{PCR efficiency} = [10^{(-1/\text{slope})}]-1$$

$$\text{Exponential amplification} = 10^{(-1/\text{slope})}$$

3.2.13 Statistical analysis

In embryogenomics, many statistical analysis have been chosen to the comparison of stages of development, as experimental effects over samples, in each case oocytes, zygotes, embryos, cultures milieu, in vivo or in vitro production among many others experimental designs, looking for statistically significant differences and most of them choose ANOVA. Because of the proper nature of the collected data, the general linear model (GLM) procedure can be applied to work ANOVA, especially for unbalanced data. For the statistical analysis among stages of development, data have been scrutinized through this procedure using least squares means with adjustment for multiple comparisons by Tukey-Kramer, using SAS software version 8.02 (SAS Institute, Inc. Cary NC, USA). Significant differences by comparison among relative mRNA abundance by stages would be indicated by letters, and means with the same letter are statistically not significantly different by alpha 0.05 that is mean showing a difference with 95% of significance.

To the parametric measure of association of the relative mRNA abundance by stages among genes was performed the correlation procedure by the Spearman product-moment correlation (Statistical Consulting Services by the University of California Los Angeles (UCLA); http://www.ats.ucla.edu/stat/sas/output/corr.htm).

4 Results

4.1 Results of molecular analysis by polymerase chain reaction

In the present study as previously was exposed, to standardize the molecular biology protocols, the expression of such homeobox bovine candidate genes was previously identified in adult bovine tissues like intestine, as well as in single or pooled samples of recollected postimplantation embryos as a tissue target and economical manner.
By using degenerated primers, corresponding to the highly conserved region of the homeodomain, were cloned and sequenced presumptive bovine EST´s for Cdx1 and HoxB9 genes, a presumptive EST corresponding to the homeodomain sequence of the *Homo sapiens* HoxA7, and an EST high homolog to the *H. sapiens* HoxB6.
Here it was tried to amplify the 3' or 5' tails of Cdx1 and Cdx2 from the found EST´s corresponding to the homeodomain region, as early was made by Hu et al. (1993) and James et al. (1994) respectively. But from both RACE-PCR reactions were only sequenced two short fragments of the homeobox, both containing the universal primer at the 5´ end. Such RACE-primers were previously tested using reverse-transcribed bovine mRNA. Using these RACE-primers were amplified and sequenced an 113nt amplicon of Cdx1 and a 101nt amplicon of Cdx2, both 100% homolog to the original EST´s.
By using GS anchored-primers was sequenced a fragment of 900 nt corresponding to the Cdx1 gene. For this was used a sense primer designed from the Cdx1 EST´s sequenced from degenerated primers and an anchored-antisense primer using the *Bos Taurus* EST locus BM446028, sequence found by blasting the mRNA sequence corresponding to *H. sapiens* CDX1 gene (XM_003791.1) and choosing the EST database limited to the specific entrez query for *Bos Taurus* [ORGN].

4.2 Results of primer design by choosing homologous fragments

Two pairs of primers were designed for each gene (Cdx2, HoxD1, HoxC9, HoxB9, HoxA1 and HoxA3) after multiple alignment of mRNA sequences from various species. Thus by choosing homologous fragments were obtained two possibilities to amplify transcripts.

The homologous fragments were used as primers (homologous primers). By which were amplified and sequenced presumptive EST´s corresponding to the Cdx2 gene (201 nt) 95% homolog to *H. sapiens* sequence (Y13709.1). It was found an amplicon (202 nt) corresponding to HoxC9 with 95% of homology to *Ovis aries* Hoxc9 mRNA sequence (U61980), 92% to *Mus musculus* Hoxc9 mRNA sequence (NM_008272), 92% *H. sapiens* HoxC9 mRNA (BC032769), and 99% with the *Bos taurus* cDNA clone (CN437384) located in the *Bos taurus* chromosome 5 (NW_931493.1). Also was found an EST (98 nt) corresponding to HoxB9. It is 97% homolog to the 5' of the *H. sapiens* HOXB9 mRNA sequence with BLAST accession NM_024017 and located in the *Bos taurus* chromosome 19. Because both possibilities of the designed homolog primers for HoxA1 and HoxA3 genes were not able to amplify any product after PCR, such genes were discarded during subsequent real-time PCR analysis.

Using homolog primers by HoxD1 was not possible to amplify any PCR amplicon from mRNA at the beginning. But by using gDNA as template was amplified a fragment (440 nt) 90% and more than 98% homologous to *H. sapiens* BC014477 and the predicted sequence by *Bos taurus* XM_597451 respectively. This sequence contains 98 nucleotides that not match to the *Bos taurus* predicted sequence (XM_597451). According to the publication of Tenney et al. (2004), this should not be considered as a putative intron, because it is not normal by mammals. Such small fragment (98 nt) matches by blasting to a *H. sapiens* HoxD1 gene sequence (87%; AF202118), as well as to a *Pan troglodytes* mRNA sequence (87%; XM_515931). That situation could be analysed in the future for such gene and their activity.

Without such small fragment (98 nt), the sequenced fragment matches in a 100% to the predicted *Bos taurus* sequence (XM_597451) and to the EST previously found employing degenerated primers. It is homolog with the 3´ end of the homeodomain. Our fragment have shown to be positioned in the *Bos taurus* chromosome 2 (NW_930852.1) by the BLAST hits in the cow genome using the Entrez Genomes MapViewer.

Here has been analysed an EST high homolog with HOXD4 gene, the fragment of 203nt presented 100% of homology with an EST from *Bos taurus* (AW486114) mRNA from pooled tissue from embryos at day 20 and 40. Such fragment correspond to the chromosome 2 (NW_930852) of *Bos taurus* genome as other paralogous members of the same group. Both are 93% homolog to Human HOX 5.1 gene (X17360) with name synonyms HOXD4, HOX4, HOX4B, HHO.C13, HOX-5.1 and Hox-4.2 (Horan et al.

1995, Ponsuksili et al. 2001a, b). On this work the fragment was used to be analysed as a presumptive fragment of the HOXD4 gene, since its homology (93%) to the *H. sapiens* HoxD4 (HOX 5.1) gene. The fragment used for real-time PCR at the present matches 100% with the *Bos taurus* DNA sequence (AAFC01407085), which is the source of the predicted *Bos taurus* similar to Homeobox protein Hox-D4 (Hox-4.2 or Hox-5.1) mRNA sequence (XM_590969) having a high homology, more than 90%, with *H. sapiens* HOX 5.1 gene by blasting.

With the aim to analyse the HoxB7 gene, *Bos taurus* sequences with accession number AF200721 and NM_174342 were used to design the GSS primers to amplify a 247 bp fragment of mRNA, which include a possible intron of approximately 1751 nt of gDNA (NW_621522) (figure 4.1). In the last time such gene has been located in the bovine chromosome 19 (NW_929500 and NW_929500.1) of the cows genome using the entrez genomes MapViewer by the BLAST hits (Bostrom et al. 2000).

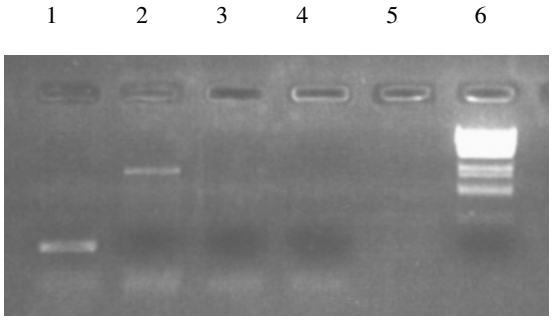


Figure 4.1 HoxB7 primer used for PCR and tested by pool of cDNAs from preimplantation embryos (1), gDNA (2) and negative control (3, 4) as templates, it is possible to see the amplification of an intron of 1751 nt approximately. PCR reactions were run in a Bio-Rad I cicler thermo controller, after that 15µl of PCR products were loaded with 1µl loading buffer and electrophoresed in 1% agarose-10X TAE buffer gel with 1% EtBr (10 mg/ml), at 125 V for 25 min. Like marker were used 5µl (500 ng/µl) of Lambda Eco 91I (Bst) (6).

Furthermore, since the previously designed primers should not be used for real-time PCR analysis, due to their origin of homolog fragments. From such EST´s were attempted to design primers for the quantification of the expression in order to work with heterologous fragments to choose GSS primers. Therefore by some gene analysis

were used homolog fragments as primers or even primers designed from the homeodomain sequence as possible (Rizos et al. 2004). Thus using bovine EST´s derived from mRNA and PCR experiments sequenced in our institute as well as from bovine sequences derived from database of NCBI corresponding to such EST´s sequenced by Ponsuksili et al. (2001a, b), GSS-primers were designed to the quantitative real-time PCR analysis of mRNA expression. These in order to avoid false positive amplification, as well as primer complementarities, like secondary structures or primer dimer, together with the two-step protocol and using of the SYBR Green DNA binding dye (Wong and Medrano 2005).

4.3 Homeobox-gene expression by real-time PCR system

PCR amplicons were amplified and sequenced corresponding to Cdx1 (154 nt; 100% homolog to *Bos taurus* XM_589247, 95% to *H. sapiens* NM_001804.1 and human CDX1 gene U51095), Cdx2 (114 nt; 95% to *H. sapiens* CDX2 Y13709), HoxD1 (136 nt; 100% to predicted *Bos taurus* HoxD1 XM_597451 and 90% to *H. sapiens* HoxD1 BC014477), HoxD4 (206 nt; 100% to *Bos taurus* AW486114 and 93% *H. sapiens* HOX 5.1 gene for HOX 5.1 protein X17360), HoxB7 (347 nt; 100% to *Bos taurus* AF200721 and NM_174342 previously commented), HoxC9 (182 nt; 100% to *Ovis aries* U61980, and 95% to *H. sapiens* BC032769), and HoxB9 (211 nt; 100% to *Bos taurus* CR852856, 99% to *Bos taurus* CN437384 and 95% to *Ovis aries* U61980) (figure 4.2).

They were the homeobox genes desired to be quantified and so establish their expression pattern; as well as by Histon A 2FZ gene (corresponding to 148 nt of H2AFZ, X52318 and NM_174809) (Fair et al. 2004a, b, Lonergan et al. 2003b, McGraw et al. 2003, Pfaffl et al. 2004, Robert et al. 2002).

Amplicons earlier cleaned, ligated and cloned, as previously explained, were used to perform serial dilutions of purified recDNA-GS and the establishment of standard curves to calculate the C_T value of the beforehand reverse transcribed mRNA as a pre-established process previously described (El-Halawany 2003, Mamo 2003, Schneider 2003, Tesfaye 2003, Tesfaye et al. 2004), which related with the molecules amount of these serial dilutions gave the possibility to estimate the quantity in the cDNA used as template. Also using such clone recDNA-GS serial dilutions, primer specificity and

quantity as well as annealing temperature and melting sets for real-time PCR conditions were standardized (Larionov et al. 2005, Wong and Medrano 2005).

Following the methods mentioned above, the quantitative expression profiling was established for Cdx1, Cdx2, HoxB7, HoxB9, HoxC9, HoxD1 and HoxD4 bovine candidate genes in the immature oocyte, in vitro matured oocyte, in vitro produced preimplantation developmental stages and some of early postimplantation developmental stages (21, 25, 32, and 39 dpi) of cattle embryos, as well as in fetal and adult gut like a positive control by a real-time PCR analysis.

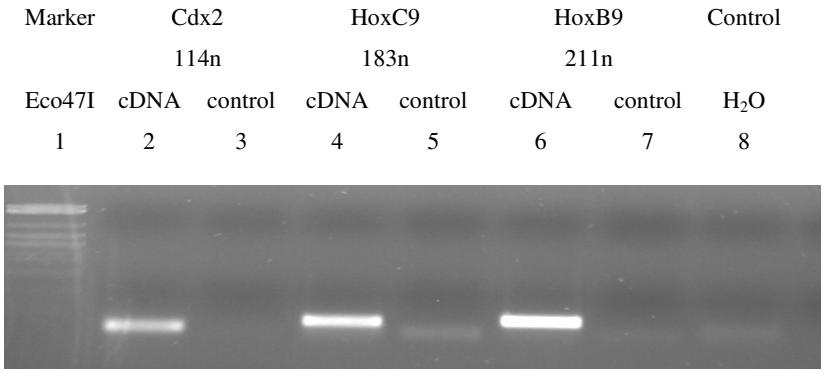


Figure 4.2 Example of PCR reactions, they have been run in a PTC-100 ™ programmable thermal controller; MJ Research, Inc. thermo cycler, after that 5µl PCR products loaded with 1µl loading buffer have been run in 1% agarose gel and 4μl EtBr 1% (10 mg/ml) for each 100 ml 10X TAE buffer, at 125 V for 25 min. Like marker was used 1µl (500 ng/µl) of Lambda DNA/ Eco 47I (AvaII).

Because the amplification efficiencies and exponential amplification of the real-time PCR reactions, results quantified in the real-time fluorescence of the SYBR green II reagent were considered satisfactory (see table 4.4.1).

Table 4.4.1 Real-time PCR and ordinary linear function characteristics from the standard curve employed to estimate the quantity from serial dilutions.

Gene	**H2AFZ.**	**Cdx1**	**Cdx**	**HoxB**	**HoxB**	**HoxC**	**HoxD**	**HoxD**
Amplicon	148 nt	154 nt	182	347 nt	213 nt	182 nt	136 nt	203 nt
PCR Efficiency	0.90	0.92	1.07	0.77	0.74	0.74	0.94	0.95
Exp. Amp.	1.90	1.92	2.07	1.77	1.74	1.74	1.94	1.95
Slope	-3.58	-3.53	-3.16	-4.03	-4.14	-4.14	-3.48	-3.46
Intercept	36.20	33.69	37.68	43.4	43.12	43.12	36.82	38.03
R^2	0.9994	0.998	0.993	0.9949	0.9978	0.990	0.9982	0.9902

Real-time PCR analysis was separated in one way to the oocytes and preimplantative stage, and in a second way to the postimplantative stages. These according to the RNA source and extraction protocol performed. For this taking in both ways the lowest relative abundance value for each one genes quantification as the lowest fold (1-fold) to be employed like calibrator for each way comparing the relative expression level among development stages. As previously established, here were not observed high differences in the outcome of the expression profiling by using the relative quantification value expressed as $2^{-\Delta\Delta C_T}$ and quantity analysis after the normalizing by H2AFZ, the expression profile was highly similar (Wong and Medrano 2005).

The gene members Cdx1 (figure 4.3-A; Freund et al. 1992) and HoxD4 (figure 4.3-B; Pitera et al. 1999) are molecular markers already characterized in embryonic and adult intestinal epithelium in human and mouse. Therefore the semi quantitative analysis by real-time PCR was applied as a positive control of gut expression at the embryo-fetal transition in a recollected identified as more than 60 dpi embryo, as well as in adult prececal gut (jejunum) and postcecal gut (colon) cells as the homeobox tissue specific expression.

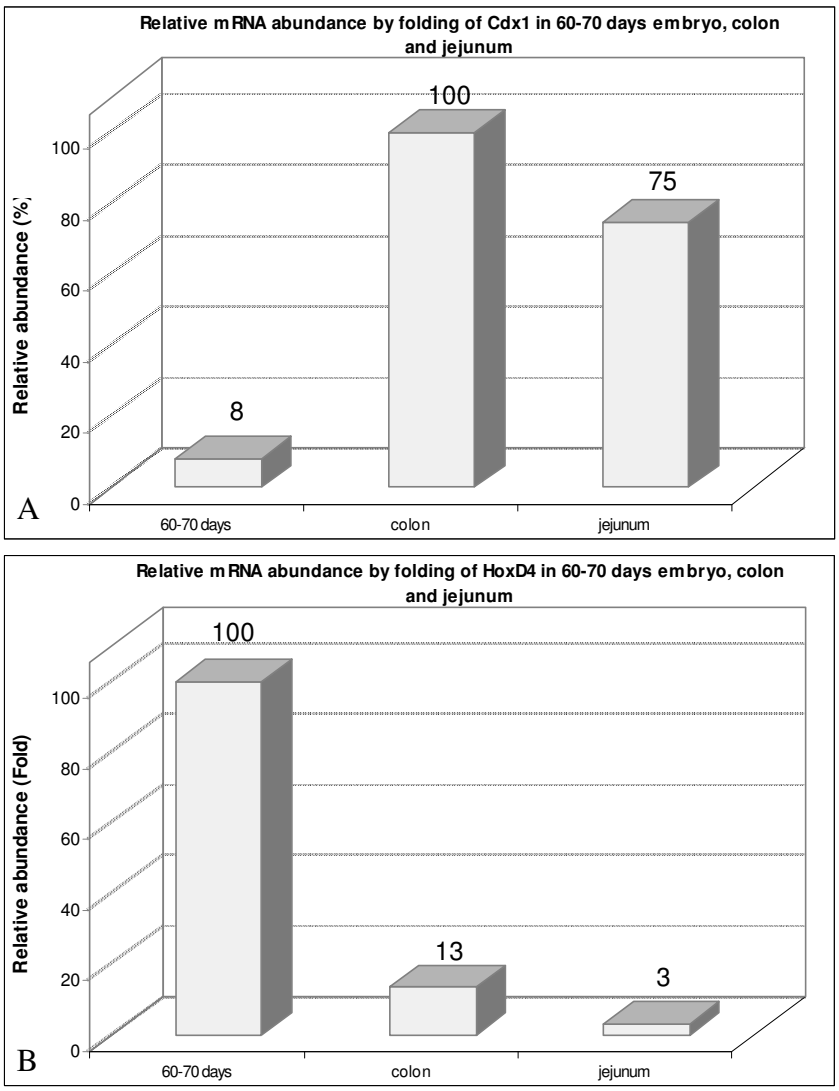


Figure 4.3 Relative abundance of Cdx1 (A) and HoxD4 (B) transcripts in intestinal cells at more than 60 dpi embryo, adult colon and jejunum, resulting as % of the highest transcript relative abundance.

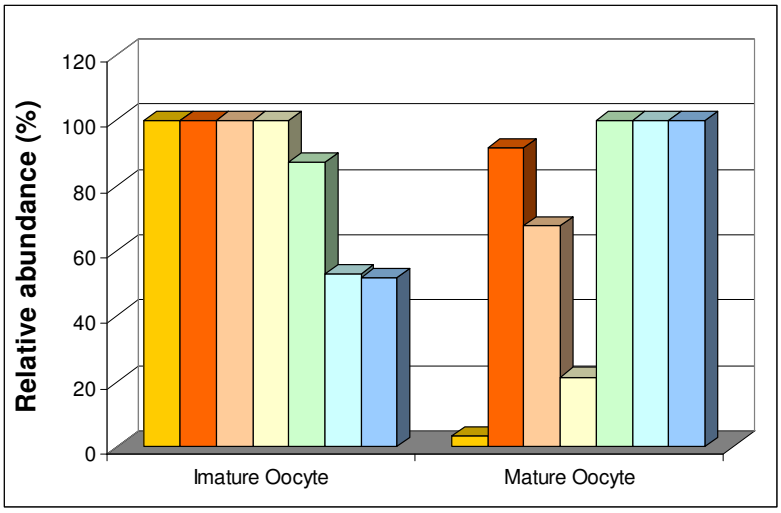
The highest relative abundance (100%) for Cdx1, Cdx2, HoxB7, and HoxB9 transcripts was observed at immature oocyte (IMO). For the members of the fourth cluster, HoxD1 and HoxD4, as well as by HoxC9 gene the highest relative abundance (100%) was observed until the mature oocyte (MO). A decline of around a 54% by fourth cluster members and 85% by HoxC9 of the highest relative abundance to the immature oocyte (IMO) was observed (figure 4.4-A). At the mature oocyte stage (figure 4.4-A), mRNA abundance for Cdx1 transcript has been resulted relative low in 6%. At this stage was observed a relative abundance for HoxB7 and HoxB9 transcripts of 62% and 27% respectively, these according to the highest relative abundance observed by IMO.

After fertilization, the highest relative abundance (100%) for all transcripts was observed in the 2 cells stage. At preimplantative stages, it was found a decreasing trend in the gene expression until their lowest level that for Cdx2, HoxB7, HoxB9 and HoxC9 transcripts was observed in the 16 cells stage. For the HoxD1 transcript, the lowest level of expression was found in the morula stage. The lowest level of Cdx1 and HoxD4 transcripts was found in the blastocyst stage (figure 4.4-B).

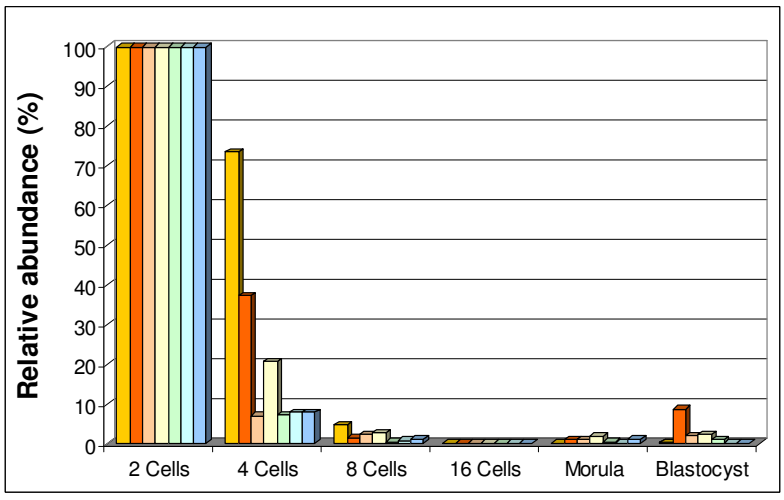
An evident increase in the expression level of Cdx2 transcript was found in the morula stage that clearly increases until a 12.34% at the blastocyst, this according to the relative abundance observed in the 2 cells stage. HoxB7, HoxB9 and HoxC9 transcripts presented a soft increasing in the expression level until the blastocyst stage showing respectively a 2.56, 2.93 and 1.48% with respect to the expression to the 2 cells stage (figure 4.4-B). This condition was not similarly observed for HoxD1 or HoxD4 transcripts, which were gradually downregulated until the morula and blastocyst stages respectively (figure 4.4-B).

In the postimplantation stages examined, a gradual increase in the expression level of Cdx1 was observed, from 21 dpi rising to be highest at 39 dpi. The relative abundance for the rest of the analysed transcripts in these postimplantation stages was relatively low at 21 dpi to turn lowest at 25 dpi. HoxB7 and HoxD4 transcripts were progressively abundant after 25 dpi to rise highest at 39 dpi similarly as Cdx1 transcript. Transcripts level for HoxB9, HoxC9 and HoxD1 was highest until 32 dpi, which was reduced at 39 dpi in an 87.46, 37.12 and 15.86% respectively (figure 4.4-C).

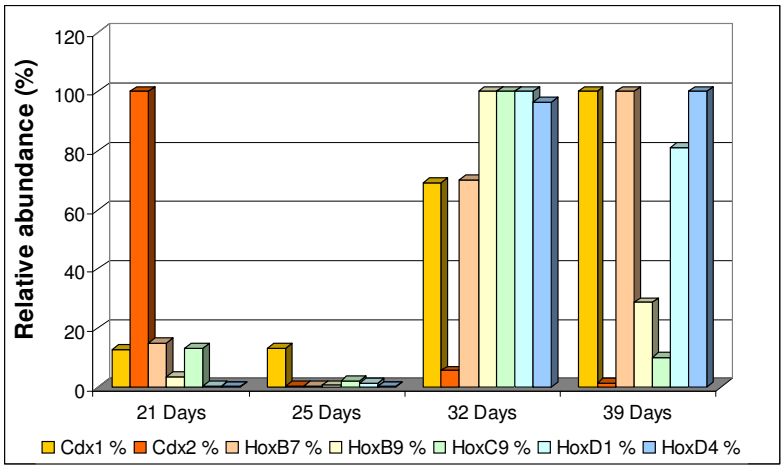
The transcript of Cdx2 gene presented a different expression profiling, since it was observed to be highest (100%) at day 21 dpi and then downregulated until a 0.84% at 25 dpi, with a softly rise of 3.93% at 32 dpi and again dropped to a 0.98% at 39 dpi (figure 4.5).



(A)



(B)



(C)

Figure 4.4 Relative expression level of homeobox transcripts as % ($2^{-\Delta\Delta C_T}$ analysis) relative to the highest relative expression for each gene throughout immature oocytes, in vitro matured oocytes (A), in vitro produced pre-implantative stages embryo (B), and postimplantative stages embryo (C).

Establishing the expression pattern of bovine Cdx2 gene, the transcript relative abundance after insemination was detected at a highest level in the 2-cell (154-fold). And then was observed a gradual degradation until the lowest level at 16-cell stage (1-fold). After that, was detected a gradual increase in the early morula and blastocyst (2-fold and 19-fold respectively) (figure 4.5).

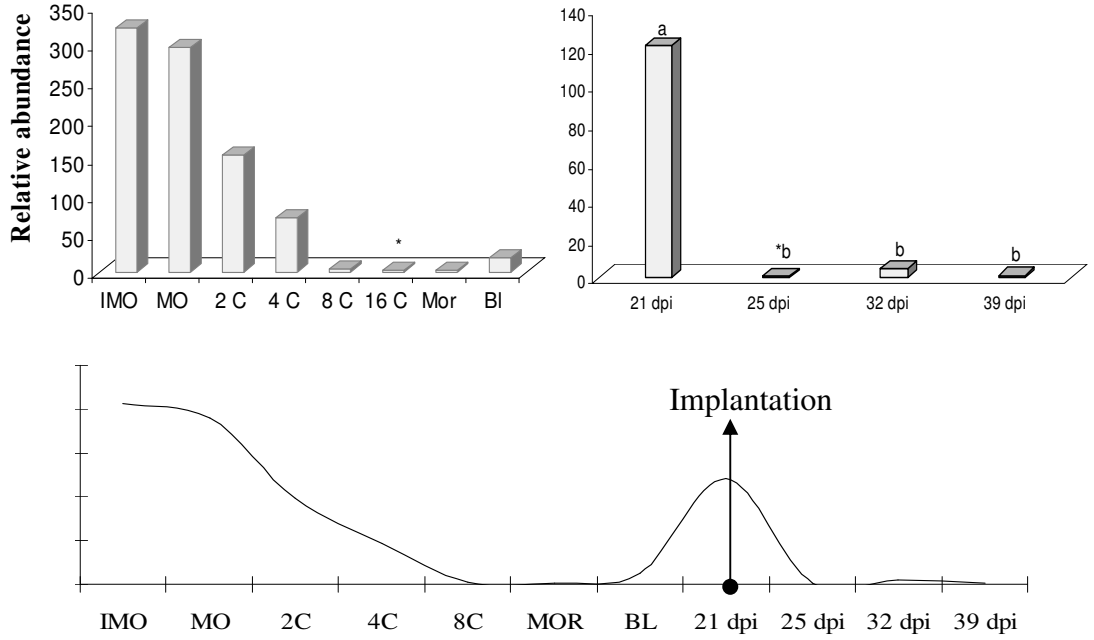


Figure 4.5 Expression pattern of Cdx2 gene by using relative mRNA abundance to the highest expression detected (IMO) as 100%. Normalization by H2AFZ gene was performed separated in two independent forms by oocytes, preimplantation, and postimplantation, employing 16 cells and 25 dpi as calibrators respectively because their lowest relative abundance.

The Cdx2 transcript was detected at a highest level at 21 dpi (121-fold), but the transcript level was drastically downregulated at 25 dpi (1-fold). It shown a lightly increase at 32 dpi to turn downregulated at 39 dpi (figure 4.5).

Below is presented the analysis of the transcript relative abundance resulted from the normalization of the homeobox genes for pre- and postimplantation stage embryos represented as folds of the lowest expression level per stage (figure 4.6).

On this analysis, it is possible to see that some transcripts at the immature oocyte stage were found to be highly abundant, as it has been observed to the transcripts of Cdx1 (9599-fold), HoxD4 (8582-fold) and HoxD1 (3143-fold) genes. A moderate to a low relative mRNA abundance was observed by HoxB7 (1038-fold), HoxC9 (809-fold), HoxB9 (456-fold), and Cdx2 (322-fold) transcripts (figure 4.6 left).

Mature oocyte exhibited the highest relative mRNA abundance of HoxD4 (15656-fold) and HoxD1 (5817-fold); and fallowed by Cdx1 (560-fold) and HoxC9 (780-fold); the lower mRNA relative abundance was found in Cdx2 (296-fold), HoxB7 (648-fold) and HoxB9 (124-fold).

The relative mRNA abundance analysis of Cdx1, HoxB7, HoxB9, HoxC9, HoxD1 and HoxD4 transcripts of the in vitro produced preimplantative stage embryos after implantation is shown in the figure 4.6 (left).

In this analysis, Cdx1 was abundant at highest level in the two-cell stage (1746-fold). In the four-cell stage, Cdx1 transcript was reduced at a level of 958-fold, and it was drastically reduced at a level of 14-fold in the eight-cell stage, to be gradually down regulated until its lowest level in the blastocyst stage (1-fold).

HoxB7 was abundant at highest level in the two-cell stage (511-fold). This transcript was drastically reduced in the four-cell stage (45-fold), after that was reduced at a level of 15-fold in the eight-cell stage to be downregulated until the 1-fold level in the sixteen-cell stage. HoxB7 was observed in the morula and the blastocyst stages at 7 and 13-fold levels respectively.

The transcript relative abundance by HoxB9 and HoxC9 transcripts was similar as by HoxB7. In the two-cell stage were detected at the highest level (150- and 278-fold respectively). HoxB9 and HoxC9 transcripts were reduced at levels of 32- and 26-fold respectively in the four-cell stage, and were downregulated in the eight-cell stage (5- and 2-fold respectively) until the 1-fold level in the sixteen-cell stage. Both transcripts, similarly as by HoxB7, were increased in the morula and the blastocyst stages at 3- and 4-fold levels respectively by HoxB9, and 2- and 4-fold levels respectively by HoxC9.

The HoxD1 was markedly abundant in the two-cell stage (1838-fold), it was significantly reduced in the four-cell stage (178-fold), and was gradually reduced at 18- and 4-fold levels (eight- and sixteen-cell stages respectively), until the lowest level at the early morula (1-fold). It shown a lightly increase at the blastocyst stage (2-fold).

The highest transcript abundance after insemination was detected in the HoxD4 transcript in the two-cell stage at a level of 12215-fold. Its relative abundance was markedly declined in the four-cell stage (1089-fold), and drastically declined in the eight-cell stage (164-fold). In the sixteen-cell stage, HoxD4 was increased in 402-fold and at the morula was again declined (167-fold). Likewise as the Cdx1 transcript, the lowest relative abundance level by HoxD4 was observed in the blastocyst stage (1-fold).
Below is presented the analysis of the relative mRNA abundance to the lowest expression by the postimplantation stages (figure 4.6 right). The relative mRNA abundance resulted from the semi quantitative analysis and H2AFZ gene normalization by the postimplantative stages recollected embryos at 21, 25, 32, 39 days after insemination was 1, 2, 5 and 8-fold by Cdx1; 26, 1, 95 and 129-fold by HoxB7; 6, 1, 90 and 33-fold by HoxB9; 7, 1, 39 and 6-fold by HoxC9; 1, 2, 359 and 399-fold by HoxD4; 1, 4, 173 and 152-fold by HoxD1.
Cdx1 has shown a progressive risen trend of mRNA expression similarly as HoxB7. Divergent situation was presented by Cdx2 as explained before, given away a relative high mRNA abundance at 21 dpi (121-fold) after insemination to abruptly decline until 1-fold at 25 dpi with a minute rising at 32 dpi (4.76-fold) and turning down at 39 dpi (1.19-fold).
The expression profile of HoxB9 and HoxC9 genes seems to be similar showing the lowest relative abundance at 25 dpi (1-fold) and the highest at 32 dpi (89.89 and 38.91-fold respectively), declining at 39 dpi (33.36 and 6.17-fold respectively).
The expression profile of HoxD1 and HoxD4, both showed the lowest relative mRNA abundance at 21 dpi (1-fold), both increased at 32 dpi (173.25 and 359.20 -fold). At 39 dpi HoxD1 appears declined (151.51-fold) and HoxD4 increased (398.79-fold) respectively.

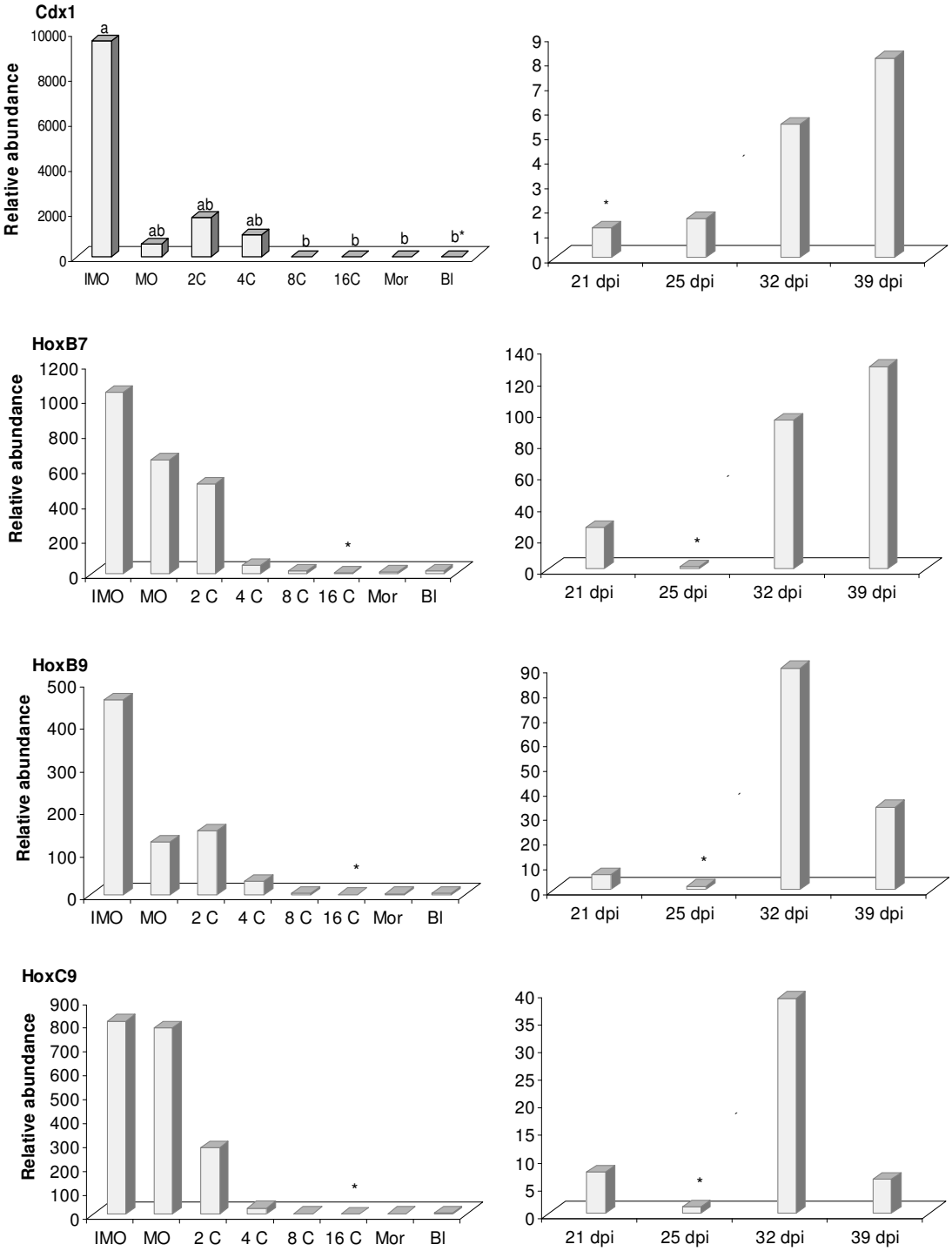


Figure 4.6 mRNA relative abundance of Hox-gene transcripts in the preimplantative (left) and postimplantative (right) stages of the embryo by using lsmeans of the GLM procedure by SAS. The lowest transcript abundance detected was used as calibrator (*). Bars with same superscripts or without are not significantly different ($P < 0.05$) by Tukey-Kramer lsmeans analysis.

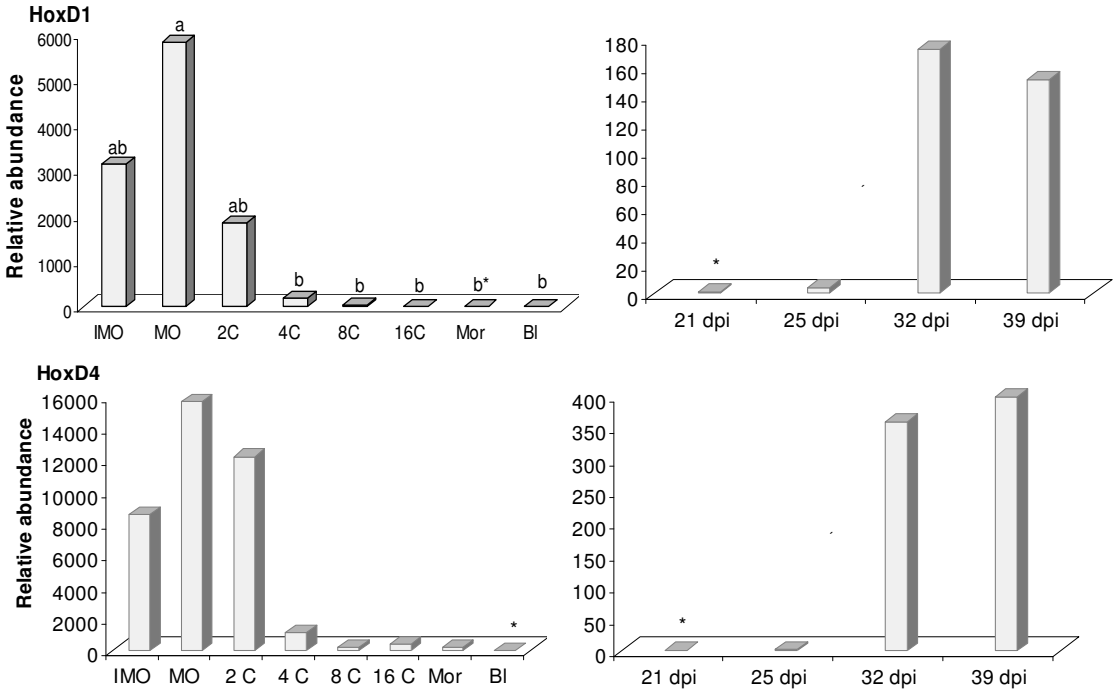


Figure 4.6 (cont.) mRNA relative abundance of Hox-gene transcripts in the preimplantative (left) and postimplantative (right) stages of the embryo by using lsmeans of the GLM procedure by SAS. The lowest transcript abundance detected was used as calibrator (*). Bars with same superscripts or without are not significantly different (P< 0.05) by Tukey-Kramer lsmeans analysis.

5 Discussion

Our goal has been aimed to do a molecular examination of these homeobox family members, which are differentially expressed during the early embryonic development and periimplantation. With this purpose, we would like to enrich our group of knowledge in the molecular reproduction, which helps to understand these natural or abnormal developmental phenomena, frequently caused by the pressure of the oocyte or the embryonic surroundings (Gandolfi et al. 2005). Because such stress conditions have been seen to drive placental defects, postnatal oversize or overweight (LOS), as well as organs failures in the offspring, still as adult diseases (Young et al. 1998). On this hand a lot of effects in the molecular biology could be related with these developmental mechanisms involved in the mammalian reproduction (McEvoy et al. 2001, 2003, Farin et al. 2006, Laurincik et al. 2003, Lonergan et al. 2003a, b, 2004, 2006, Wrenzycki et al. 2005b, Ecker et al. 2004).

Many studies have been abundantly realized in the Homeobox mRNA and protein expression around implantation, as well as during primary differentiation events in the early mammalian development, also after blastocyst expansion, gastrulation, primitive streak formation, embryonic disk proliferation, organogenesis and limbogenesis (Beck et al. 1995, 1999, 2000, Charité et al. 1998, Chawengsaksophak et al. 1996, 1997, 2004, Deschamps et al. 1999, Manzanares and Krumlauf 2000). All these works have been focussed to determine the expression of Hox genes combination as well known as the Hox-code. This network appears to be very important in the spatial and temporal co-linearity during the early body patterning (Deschamps et al. 1999). Still, a lot of works have been directed on the mRNA and protein expression of the caudal members (Cdx) transcription factors as regulators of the Hox-gene expression among other genes, recently well reviewed by Deschamps and van Nes (2005) and Grier et al. (2005).

The A-P patterning and the body-tissue identity of the mammalian embryo, as by others living beings, are directed by the interaction of the nuclear transcription factors as the homeodomain related proteins activity (van Nes et al. 2006, Pilon et al. 2006). The stress by surroundings appears to be highly related to placental and body-tissue development defects (Hall et al. 2005).

As previously shown, mRNA expression of some members of the known homeobox or homeodomain family of transcription factors has been detected by RT-PCR techniques

in unfertilized oocyte and pre-embryos of mammals as mouse (Pitera et al. 2001, Ralston and Rossant 2005, Strumpf et al. 2005), and human (Verlinsky et al. 1995, Kuliev et al. 1996, Adjaye et al. 1999, Adjaye and Monk 2000).

5.1 Molecular analysis by polymerase chain reaction

In the present study to standardize our molecular biology protocols, at first the expression of such homeobox bovine candidate genes was previously identified in adult bovine tissues like intestine, as well as in single or pooled samples of recollected postimplantation embryos as a target tissue and economical manner.

As the way early employed by Ponsuksili et al. (2001a, b), we used degenerated primers taken from Murtha et al. (1991), which correspond to the highly conserved region of the homeodomain. Here were cloned and sequenced presumptive bovine EST´s for Cdx1 and HoxB9 genes analogous to these discoveries by Ponsuksili et al. (2001a). The presumptive EST corresponding to the homeodomain sequence of the *Homo sapiens* HoxA7, previously was reported to be expressed in human unfertilized oocytes and in the preimplantation development of embryos, as well as by embryos with chromosomal aneuploidies (Adjaye and Monk 2000, Kuliev et al. 1996, Verlinsky et al. 1995). In addition, the presumptive EST high homolog to the *H. sapiens* HoxB6 has been reported to be expressed by mouse and human ovary and oocytes (Villaescusa et al. 2004), as in trophoblast cells (Amesse et al. 2003, Zhang et al. 2002). Thus these ESTs, high homologous to genes previously found at very early mouse, human and now bovine oocyte and early embryonic cells, suggest their participation on the bovine oocyte maturation, early bovine embryonic development, periimplantation, oogenesis, and further developmental stages.

Here we tried to amplify the 3' or 5' tails of Cdx1 and Cdx2 from the found EST´s corresponding to the homeodomain region, as early was made by Hu et al. (1993) and James et al. (1994) respectively. RACE-PCR reactions were performed using the BD SMART™ RACE system (BD Biosciences, Clontech) and were run with primers designed following the recommendations of the BD SMART™ RACE cDNA amplification kit user manual. Such RACE-primers were previously tested in reverse transcribed bovine tissue mRNA through RACE-cDNA and oligo-T7 primer cDNA systems used as template. Using these RACE-primers were amplified and sequenced an

113nt amplicon of Cdx1 and a 101nt amplicon of Cdx2, both 100% homolog to the original EST´s. But from the RACE cDNA the only one product amplified was a short fragment of the homeobox containing the universal primer at the 5´ end. It could be due to some characteristics in the nucleotide chain as appears to be 64.39% GC by Cdx1 and 65.06% GC by the Cdx2 bovine putative genes.

In our experience, it was not easy to find such primers for all desired genes to be analysed, may be for the natural evolutionary homology among the gene family, even because to the high proportion of G or C bases observed inside of these sequences (Andersson et al. 1996, Pennacchio and Rubin 2001). Bovine Cdx2 gene mRNA sequence from an "in silico" analysis probably present 14.71% T, 20.21% A, 30.78% G and 34.28% C. This is a frequent situation that also affects the amplification process and generally the cloning at the cell transformation (Buckland et al. 2005).

In general our results are in agreement to these of previous studies in other species (Adjaye et al. 1999, Adjaye and Monk 2000, Kuliev et al. 1996, Pitera et al. 2001, Ralston and Rossant 2005, Strumpf et al. 2005, Verlinsky et al. 1995), which have shown the mRNA or protein expression at the early pre- and postimplantation, in adult tissues, and still by bovine (Ponsuksili et al. 2001a, b).

The 900 nt of bovine Cdx1 mRNA here sequenced shown a high homology with the *H. sapiens* (NM_001804), *Rattus norvegicus* (M91450) and *Mus musculus* (NM_009880) CDX1 sequences. It is 100% homolog with the actually predicted Cdx1 mRNA *Bos taurus* sequence (XM_589247). This CDX-1 (XM_589247) sequence has a length of 523 nt and has been derived by automated computational analysis using the gene prediction method (GNOMON) including similarity to 5 ESTs and 27 proteins. Both sequences have more than 95% of homology with their orthologous gene in human.

Consequently in this work, bovine specific mRNA sequences were achieved by means of PCRs using different systems, as was done by primers designed from homolog fragments. They would be used as characterized ESTs, which together with the sequence bovine genome updated from the NCBI data bank, have helped to detected and construct the presumptive gene sequences of Cdx1 and Cdx2 genes by "in silico" analysis. Until now an immense quantity of sequences data on homeodomains have been accumulated from different species analysed during the past 20 years. All this information is of main importance for the study of the reproduction, embryology,

development, evolution and genetics giving a special impact to this gene family (Gehring et al. 1994, Chawengsaksophak et al. 2004).

On this form, by combining our experimental results, and helped with computer modelling in these “in silico” ways, were determined elements (Ko 2004), which could be use to determine the gene and protein expression of homeobox transcription factors and signalling pathways or protein synthesis in vitro and folding studies for small proteins function (Clementi and Plotkin 2004, Marchler-Bauer and Bryant 2004, Noireaux et al. 2003, Sawasaki et al. 2002). Sequences here found present similar elements as these CDX1 and CDX2 human sequences (Mallo et al. 1997) and murine cdx-2 (James and Kazenwadel 1991). Both are highly homologous among species and Cdx members (Drummond et al. 1997).

These elements are of principal importance to determine protein properties and functional activities or molecular and biochemical points of action. Knowing the functional form of the protein is of principal importance for the molecular biology (Clementi and Plotkin 2004). For example, a functional discrimination of Hox gene members has been observed. For this appears as a key the protein-protein interaction in different regions. Given that a high specificity is needed by their biochemical interaction with heterologous proteins in vivo. Here the protein folding could help to appreciate such molecular interactions (Sprules et al. 2003, Bondos et al. 2004).

5.2 Quantitative homeobox-gene expression by real-time PCR

The present work shows the whole pattern of mRNA expression of some representative candidate genes of the homeobox gene family. All of them were identified in the immature oocyte, in vitro matured oocyte and the preimplantative in vitro produced embryos of cattle, as well as they were presented at the postimplantation stages, early development and adult gut.

The caudal related homeobox gene members, Cdx1 and Cdx2, have been characterized as primary transcription factors in adult intestinal epithelium as well as molecular markers of mRNA and protein expression in the chickens, mice, rats and humans intestine (Freund et al. 1992, Freund et al. 1998, Geyra et al. 2002, James and Kazenwadel 1991, James et al. 1994, Kaiser et al. 2003, Subramanian et al. 1998). Therefore here has been quantified Cdx1 expression at the embryo-fetal transition in an

embryo identified as more than 60 dpi, in adult prececal gut (jejunum) and postcecal gut (colon) (figure 4.3); this similarly as the pattern of expression by mice as formerly Mallo et al. (1997) detected by CDX1 and CDX2 mRNA expression in small intestine, colon and rectum using Northern-blot analysis. Thus our results of the quantitative expression are in agreement to these previously observed by using other techniques.

James and Kazenwadel (1991) found the Cdx1 mRNA expression to be more abundant in colon as compared to the small intestine in adult mice. Here was obtained a 25% of difference between colon (higher) and jejunum. As well the HoxD4 level of expression makes evident its participation in embryonic gut development (figure 4.3). HoxD4 gene has been previously demonstrated to be needed for a correct intestinal development at the early embryonic gut differentiation as observed in the expression patterns of genes members of the HoxD paralogous groups 4 and 5 (Pitera et al. 1999).

The quantitative expression profiling of Cdx1, Cdx2, HoxB7, HoxB9, HoxC9, HoxD1 and HoxD4 bovine candidate genes expressed as percentage and the mRNA relative abundance to the lowest expression from the analysis by the real-time PCR are really an element that helps to establish the spatiotemporal mRNA expression of this gene family (Kuliev et al. 1996, Adjaye et al. 1999, Adjaye and Monk 2000, Ponsuksili et al. 2001a, b). So mRNA transcripts of some members of the homeobox containing genes analysed (Cdx2, HoxB7, HoxC9, HoxD1 and HoxD4) were characterized to be higher in matured oocyte through the zygote to embryo stages to get dropping lightly until their lowest relative abundance between the 8- to 16-cell stages, which could appear as a maternally derived transcription (Lloyd et al. 2003). These since the major transition of maternal to embryonic gene expression in bovine normally occurs during the late 16-cell stage as presented by many genes, as Lloyd et al. (2003) observed in mice by Wnt3a transcript related to the homeobox gene family.

On another form was presented by HoxB9 and Cdx1 that seem to have a different derived transcription. Both presented an increasing at 2-cells stage, but it could be not discarded the possibility to be activated for the first round of DNA replication in the minor gene activation between the 1- to 4-cell stages in bovine (Memili and Firts 1999). The maternal to embryonic transition in the cow has been characterized by a minor and a major gene activation. This minor gene activation is very imperative for further embryo development and the major gene activation between the 8- to the 16 cell stages.

Here we observed a similar trend of expression in oocyte among gene members of the fourth cluster HoxD1 and HoxD4.

Cdx proteins are gradually produced in the embryo development and upstream the Hox-genes expression pattern (Charité et al. 1998, Gaunt 2001). Here we observed a gradual decreasing of transcript levels by all hox-members from the oocyte to the zygote. And the same has been found from the two cell stage to the blastocyst. After implantation, a gradual increase of transcripts was observed. But not by Cdx2 gene that the transcript increase was observed to be early at morula.

Recently, Deb et al. (2006) characterized the protein and mRNA expression of Cdx2 in mouse oocyte and through the preimplantative development by using immunofluoresence. Cdx2 protein was not detectable in the unfertilized oocyte (metaphase II), but the Cdx2 mRNA was observed being polarized to a hemisphere of the oocyte. Here in our findings it is shown the highest quantity of Cdx2 mRNA in IMO (322-fold), after that drops at the unfertilized oocyte (metaphase II) (297-fold). Thus we consider this transcript as not abundant (figure 4.5), similarly as was found by Deb et al. (2006). Also here mRNA expression of Cdx2 seems to have an increased trend of expression after fertilization as observed by mice (Deb et al. 2006). The trend of expression of Cdx2, HoxB7 and HoxC9 bovine transcripts has been found to be very similar as the protease serine 23 (SPUVE) and chloride intracellular channel (CLIC1) bovine transcripts early analysed by El-Halawany et al. (2005).

Degrelle et al. (2005) analysed Cdx2 mRNA expression in elongating bovine blastocysts between 7 and 17 dpi. They found a weak expression in the whole spherical blastocyst stage (7 dpi) and an increasing expression at ovoid stage (12dpi), but only in the mural trophoblast of the extra embryonic tissues; the expression in the polar layer was not detectable. A highest Cdx2 mRNA expression was observed in the extra embryonic tissues at the early filamentous blastocyst (17 dpi) for the trophoblast lineage (Degrelle et al. 2005). We detected an increasing of expression from early morula to blastocyst, and the highest Cdx2 expression was found at 21 dpi considered as implantation day.

5.3 Early embryonic development, surroundings, and protein pathways

As early well reviewed (Ko 2004), these elements help to the understanding of many biological situations in the normal development, diseases or physiological complex disorders, as these presented in cattle at the periparturient period affecting implantation (Lonergan et al. 2003a, b, 2004, Wrenzycki et al. 2005a, b). Still, they help us to understand the posterior development having effects in adult animals, as these detected in humans and rodents (Farin et al. 2004, Ecker et al. 2004, McEvoy et al. 2003).

Evidently all these elements can be applied to clarify many biological phenomena carried out during assisted reproduction procedures, since changes detected in gene expression can be their origins (Adjaye et al. 2005, Wrenzycki et al. 2004, 2005a, b). Also the delineation of signalling pathways and expression patterns may be fundamental for the understanding of mechanisms regulating pluripotency and self-renewal in cultured ES cell lines (Adjaye et al. 2005).

Individually, Hox-gene members have multiple promoters giving rise to different transcripts to be expressed in diverse tissues (Aubin et al. 1998). So they have been classically characterized as transcriptional activators and/or repressors in order to an appropriate development of specific cells. Hox-genes centrally determine a spatial regulation in the morphogenesis, identity patterns of the body and growth (Manzanares and Krumlauf 2000, Taylor et al. 1997).

Thus in the mammalian early embryonic development, the expression of hox-genes would participate as a key of a vastly integrated pathway, the Hox-code. This network operate in the morphological regionalization arrange, together with many neighbouring genes in the same linkage group, other paralogous genes, and even nonparalogous genes localized in separate clusters (Chen and Capecchi 1999). All of them work in a positive or negative manner by up or down gene regulation, but always analogous with each other and not as an individual component. And so the integration of the pathway forms the system functioning cooperatively to a proper embryo regionalization along the body axes. Their activities must be closely synchronized in a temporal-, spatial-, and gene specific manner because their global Hox-code function (Bondos et al. 2004).

Deschamps and van Nes (2005) have discussed that the area around the hox-clusters has some DNA sequence elements, which direct the activity of the Hox-gene expression in a cell-tissue specific pattern, and could be independent in the described spatiotemporal

Hox-gene expression. But it is considered that the Hox-genes regulation at the early epiblast is independent of the Hox-genes participation later in the development. These because the hox gene expression derives from specific differentiated cells (Mitsui et al. 2003, Imakawa et al. 2004, Strumpf et al. 2005, Yates and Chambers 2005).

Here we analysed the mRNA expression of these 7 homeobox genes uncovered from oocyte, pre- and postimplantative libraries. Anyway, their protein activity in the oogenesis, oocyte maturation and after fertilization remains to be well characterized. Perhaps it is possible to study the functionality of these transcripts in bovine embryos by analysing their protein expression in situ as made by Strumpf et al. (2005), Niwa et al. (2005), and Deb et al. (2006) in mice blastocyst for Cdx2 and other hox related genes. Also the establishment of the interfering RNA (siRNA) method for gene silencing could grant us in the future for a lot of facts to decipher homeobox-gene code.

Until now no much information has been revealed with respect to the homeobox-gene activity in such stages of the early embryonic development, therefore here is a point to enrich our group of knowledge in regard to reveal the hox-code and its association with other developmental pathways as made Deb et al. (2006) by analysing Cdx2.

Using small siRNA procedures, Luo et al. (2004) observed a pronounced depression of HoxB9 transcription in chick embryos by siRNA against geminin, a protein working together with Cdt1 in the Hox-code repression. In such work they determined that HoxB7 regulate the FGF2 expression in melanoma cell models, increased expression of geminin produce depression of FGF2 expression through the regulation of HoxB7 and this process appears as concentration dependent. Furthermore Hay et al. (2004) detected that FGF2 have effects in the expression of Cdx2 and the induction of trophoblast differentiation by its application in the culture of human embryonic stem cells (hES).

Homeobox nuclear proteins could be elements to help the interaction of the milieu with cell behaviour, because they incorporate external information to produce such differential DNA bindings, so controlling the gene transcription (Bondos et al. 2004, Deschamps and van Nes 2005). They require intrinsic and extrinsic mechanism to regulate their function and their expression varies according to the surroundings.

These could be assumed for the Hox-code auto regulation through the interaction of heterologous proteins and the participation of such transcription factors as HoxA4, HoxA7, HoxB4, HoxB5, OCT-1 and OCT-4, or developmental genes as WNT11, RARE´S elements and DNA-binding proteins. Hox gene members appear to be

concentration dependent of such elements (Lickert et al. 2000, Allan et al. 2001, Aoki et al. 2003, Houle et al. 2000, 2003, Jette et al. 2004). Even, Hox-genes expression, as other transcription factors, changes as a response to the surroundings, this was showed in cell models by Marchetti et al. (2003). They observed the activation of Cdx2 expression by an acidic pH.

Later Chen et al. (2005) explained that Cdx2 expression can be up regulated by cAMP in proglucagon producing cells. Thus has been proposed that Cdx2 could be regulated in a cell-type specifically by underlying mechanisms as the second messenger cAMP via a specific pathway as Epac. Even, changes in intracellular iron levels in intestinal and colonic epithelium by implying regulatory pathways lead enhanced iron export, which could modulate CDX2 expression (Hinoi et al. 2005). But how affect all these process the oocyte maturation and the preimplantative development remains to be clarified. However, the induction of Cdx2 and other trophoblast-associated genes has been seen to be dependent on the culture conditions (Hay et al. 2004).

In contrast, this protein, as transcription factor, was early clearly detected in the nucleus and cytoplasm of the outer cells of early morula and late morula stages (Strumpf et al. 2005). Recently, Cdx2 mRNA has been detected at all cell stages during preimplantation development of mouse, from zygote to blastocyst stages (Deb et al. 2006). And according to many other works, Cdx2 has been though to be the first homeobox containing gene identified and required by Oct4/Nanog for the normal blastocyst development, since Cdx2 mutants show homeotic transformation defects and homozygous mutants of Cdx2 could cause embryonic die at the periimplantation stage (Chawengsaksophak et al. 1997, Tamai et al. 1999, Ralston and Rossant 2005, Strumpf et al. 2005).

But van Nes et al. (2006) affirmed that during placentogenesis, Cdx members operate in a redundant centrally form involved in placental morphogenesis and the ontogenesis of allantoic components of the placental labyrinth. Thus Cdx2 plays an obligatory part, assisted by Cdx4, which can take a crucial role when one Cdx2 allele is inactivated. Therefore heterozygous mutants could not die.

Cdx2 gene is expressed for TE differentiation of blastocyst (Beck et al. 1995, Chawengsaksophak et al. 2004, Goldin and Papaioannou 2003, Imakawa et al. 2004). It directs subsequent implantation, trophoblast lineage during postimplantation stages by extraembryonic membranes, placenta development, and essentially participates in the

axial elongation. So that, Cdx2 gene can now be used as a specific marker transcript in different species like mouse, human and cattle or pigs to study the proper TE differentiation. This could be employed in order to identify important molecular functions within the blastocyst, as previously this has been done by analysing colon cells (Adjaye et al. 2005). Also in recent times, it is known that such gene is really necessary for the maintenance of trophoblast stem (TS) cells as is by Oct4 (Imakawa et al. 2004).

As exposed earlier, Cdx2 mutant blastocysts fail to implant, since they can not maintain a proper trophoblast differentiation, as well such loss of Cdx2 has been linked to downregulation of Oct4 and Nanog genes in outer cells of the blastocyst resulting in outer cells decease (Adjaye et al. 2005, Niwa et al. 2005, Strumpf et al. 2005). Given that Cdx2 control the lineage-restricted expression of Oct4 and Nanog, such situation has positioned this gene in a pivotal role for proper implantation.

Hart et al. (2004) studied the homeobox members, mouse and human Nanog, to be detectable in the ICM of the blastocyst, after implantation in the proximal epiblast region of the presumptive primitive streak, extended gastrulation and remaining restricted to epiblast. Such members establish the germ cells pluripotency in embryonic gonads, as in germ cell tumour, undifferentiated embryonal carcinoma cells line and teratoma-derived cell lines. Also they are expressed in several adult tissues.

There is a high conservation between mouse and human in Nanog genes as classically in the Hox-gene family, reflecting a common expression pattern. Comparing Nanog members with Oct4, which are expressed uniformly in the epiblast and mesoderm at gastrulation, it has been observed their expression more closely to Mixl1 or Otx2. This could be taken as an evidence of other Hox-gene members involved in early embryonic patterning and cell fate determination (Hart et al. 2004).

Our results obtained from the gene expression profiling by the semi quantitative real-time PCR system are highly coincident with the information previously mentioned, as is the increasing Cdx2 and HoxB7 expression at early morula and blastocyst stages. Both findings are directly related with the expression pattern established in mouse (Ralston and Rossant 2005, Strumpf et al. 2005, Deb et al. 2006), and human (Adjaye and Monk 2000).

Also such situation has been previously well established in mouse embryos, where Cdx2 gene, as transcription factor, is expressed for a proper TE differentiation in

blastocysts during preimplantation development directing the subsequent implantation, by extraembryonic membranes, placenta and essential for axial elongation (Beck et al. 1995, Chawengsaksophak et al. 2004, Goldin and Papaioannou 2003, Imakawa et al. 2004). Thus Cdx2, as the earliest transcription factor identified so far to be involved in specification of TE fate, is required for repression of Oct4/Nanog and normal blastocyst development. Therefore this gene, as other Hox-gene members, could be related with these effects in LOS (Lazzari et al. 2002, McEvoy et al. 2003, Young et al. 1998).

The characterization of the relative abundance of all transcripts presented in this work is really amazing, because the congruence with these presented in previously works by ways totally different. These specially by Cdx1 and Cdx2, which have been shown highest at the immature oocyte (as 100% base); by mature oocyte the relative abundance of Cdx1 suffers notably drop, but by Cdx2 goes down as few as an 8 %, showing later a progressively dropping until turn lowest at the 16 cells (1-fold), similarly as HoxC9; this seems to be a typical maternal transcript storing, since its expression appears progressively raised from morula to blastocyst, as observed by HoxB7.

After implantation Cdx2 has been shown higher at the 21 dpi (100% at this stages), after that suffers notably drop as normally work such protein in other mammals. Otherwise transcript level of Cdx1 after zygote formation got raising at 2 cells (around 20 %) turning progressively low until disappears at the blastocyst to turn progressively raised after implantation, showing its probably participation after gastrulation in the establishment of the anterior-posterior patterning along the embryonic axes as by human or mouse.

Cdx1 and Cdx2 experience a reciprocal pattern of expression and their pattern of enhancer activation can be perturbed along this axis in the Cdx mutant transgenic mice (Maier et al. 2005). The trend of expression of Cdx1 and HoxB9 is highly correlated, as observed by Cdx2, HoxB7 and HoxC9; and the observed by HoxD1 and HoxD4. But really interesting are the results here presented by HoxB7 and HoxC9 compared with the results of Miano et al. (1996). They experience a high correlation in the spatiotemporal expression of the nonparalogous hox-genes HoxB7 and HoxC9 in human fetal lung, kidney, thigh brain, spinal cord, as well as in adult lung and the awesome participation of Hoxd members in the organogenesis that can be reflected after born, even in adult mammals (Zakany et al. 2001).

Furthermore, to improve the assisted reproductive technologies (ART) as in vitro maturation and production of oocyte and embryo respectively, it is necessary to characterize the expression pattern of transcription factors being pivotal for important events in the early embryonic development. These results show us that Hox gene transcription factors, especially Cdx2 and perhaps HoxB7 and other Hox members are important transcripts in the early preimplantation development, suggesting their role in in vitro development of cattle embryos being really interesting for further studies related with developmental phenomena previously observed.

In conclusion, the present study has shown that Hox-genes are active transcripts in bovine preimplantation and postimplantation stages, supporting their potential involvement in the early embryonic development as nuclear transcription regulators.

6 Summary

Bovine embryogenesis is driven by molecular mechanisms establishing the early embryonic development, but until now a lot of molecular pathways remain to be well studied. Homeobox (hox) genes encode transcription factors which regulate the expression of several target genes encoding essential proteins for cell differentiation and proliferation. The gene expression of transcription factors plays an important role in oocyte maturation, pre- and post- implantation development.

It was the purpose of the present study to investigate the expression profile of hox-gene family members in pre- and postimplantation developmental stages of bovine embryos. For this, EST´s for Cdx1, Cdx2, HoxA7, HoxB6, HoxB7, HoxB9, HoxC9, HoxD1 and HoxD4 were sequenced. After that, the expression profiling of Cdx1, Cdx2, HoxB7, HoxB9, HoxC9, HoxD1 and HoxD4 genes has been investigated using the quantitative real-time PCR system. Quantification was done in triplicate pools (each containing 10) of immature oocytes, matured oocytes, 2-cell, 4-cell, 8-cell, 16-cell, morula and blastocysts. Also the real-time PCR quantification was applied to determine the expression profiling of ex-vivo harvested embryos at day 21, 25, 32, and 39 post insemination. Cdx1 and HoxD4 gene expression of more than 60 days embryo gut cells and adult prececal and postcecal gut cells was used as positive control of transcript expression.

It was found that the relative abundance of all transcripts appears congruent among the members of the gene family. The highest relative abundance was observed for Cdx1, Cdx2, HoxB7, HoxB9, and HoxC9 at the immature oocyte stage, after that was down regulated in the later developmental stages. For HoxD1 and HoxD4, transcripts expression was abundant at higher level in mature oocytes, afterward was down regulated in the later developmental stages. After zygote stage, expression appears increased at 2 cells by HoxB9 and Cdx1 (20 %) and then transcript abundance declined progressively.

Transcripts of Cdx2, HoxB7, HoxB9, and HoxC9 were found to progressively increase after 16 cells stage until blastocyst stage. The Cdx2 transcript was found to have abundant level at day 21, which may support its potential role in embryo implantation. The relative abundance of Cdx1 was found to progressively increase at day 21, 25, 32, and 39, similarly as HoxB7. This transcript was progressively abundant after 25 dpi

rising to the highest rate (100%) at 39 dpi. The expression profile of HoxB9 and HoxC9 genes seems to be similar after implantation, both showed the lowest relative abundance at day 25 and the highest at day 32, declining to 87.46 % and 37.12% at day 39 respectively. HoxD1 and HoxD4 showed the lowest relative mRNA abundance at day 21, increasing at day 32. At day 39, HoxD1 appeared declined (15.86%) and HoxD4 was increased.

The expression pattern of Cdx2 was observed to be highly correlated to HoxB7. Interestingly, strong correlation was observed between HoxB7 and HoxC9. In general our results are in agreement to these results presented in previous studies by other species. Thus our quantitative expression is congruent to results previously observed by using other techniques as northern blot and immunoassays.

mRNA transcripts of some members of the homeobox containing genes analysed (Cdx2, HoxB7, HoxC9, HoxD1 and HoxD4) were characterized to be higher in matured oocyte through the zygote to embryo stages to get dropping lightly until their lowest relative abundance between the 8- to 16-cell stages, which could appear as a maternally derived transcription.

HoxB9 and Cdx1 seem to have a different expression pattern. Both showed an increase at 2-cell stage, but it could not be excluded the possibility to be activated for the first round of DNA replication in the minor gene activation between the 1- to 4-cell stages in bovine. The maternal to embryonic transition in the cow has been characterized by a minor and a major gene activation. This minor gene activation is imperative for further embryo development to the major gene activation between the 8- to the 16-cell stages. Here we observed a similar trend of expression in oocyte among gene members of the fourth cluster HoxD1 and HoxD4.

Cdx proteins are gradually produced in the embryo development and are upstream regulators of the Hox-genes expression pattern. Here we observed a gradual decreasing of transcript levels by all hox-members from the oocyte to the zygote. And the same has been found from the two cell to the blastocyst stages. After implantation, a gradual increase of transcripts was observed. In the Cdx2 gene, the transcript increase was observed to begin at early morula.

Recently, the characterization of the protein and mRNA expression by Cdx2 gene in mouse oocyte and through the preimplantative development shows that Cdx2 protein was not detectable in the unfertilized oocyte (metaphase II), but the Cdx2 mRNA was

observed being polarized to a hemisphere of the oocyte. Here in our findings, it is shown the highest quantity of Cdx2 mRNA in IMO (322-fold), after that it drops at the unfertilized oocyte (metaphase II) (297-fold). Thus, we consider this transcript as not abundant at such stages in comparison with Cdx1 (9599-fold), HoxD4 (8582-fold) and HoxD1 (3143-fold) genes at IMO stage. Also here, mRNA expression of Cdx2 seems to have an increased trend of expression after fertilization, as observed in mice. The trend of expression of Cdx2, HoxB7 and HoxC9 bovine transcripts has been found to be very similar as the protease serine 23 (SPUVE) and the chloride intracellular channel (CLIC1) bovine transcripts during the same stages of development. Such transcripts were early analysed in other works through the same system.

Our results have shown that Hox-genes, especially Cdx2, are important markers at early preimplantation stages and periimplantation, because their involvement in the early embryonic development. Moreover, Cdx2 is required for blastocyst development, TE differentiation and placenta formation. Therefore this gene together with other Hox members could be a potential markers which could be employed in the study of the effects observed in the large offspring syndrome (LOS).

In the study of the fertility and to improve assisted reproductive technologies (ART), as in vitro maturation and production of oocyte and embryo respectively, it is necessary to characterize the expression pattern of transcription factors being pivotal for important events in the early embryonic development. Here the results show that Hox gene transcription factors in early preimplantation stages could be important suggesting their role in in vitro development of cattle embryos.

In conclusion, the present study has shown that Hox-genes are active transcripts in bovine preimplantation and postimplantation stages, supporting their potential involvement in the early embryonic development as nuclear transcription regulators.

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I want to express sincerely my deepest thanks to my Professor Father, Prof. Dr. Karl Schellander, how granted me and my family with the opportunity to come, live and work in Germany, for the direct or indirect help to have a PhD position in the Rheinischen Friedrich-Wilhelms - University of Bonn, and of course for the place that you gave me in your heart and for the supervision of my work.

On the same form, I am profound thankfully with all the people which participate in the CONACYT (Mexico) – DAAD (Germany) program, which participated directly or indirectly by helping me to obtain the scholarship and gave me wonderful ideas to solve all the obstacles here presented.

A special thanks to Prof. Dr. Brigitte Petersen for the cooperative supervision of this work and Prof. Dr. Thomas Kutsch for the great examination, which will be reflected in my development.

I am very much thankful to Dr. Dawit Tesfaye for helping me always friendly and unconditional to realize as possible this work and to participate in my dream as researcher. The coincidence gave us the opportunity to know us, truly thank you for believe in me. On this way, I would like to express a justly appreciation to the international community of the institute for animal science for the friendly participation in this work, especially to the people from Ethiopian which helped me always, and the people from Thailand that took friendly participation in my work.

I would like also to express a profound thankfulness to all the staff of the institute for animal science of the University of Bonn, which have always helped me in an unconditional and truly form to have a warmest and cordial time as guest here, of course for your participation in this work, thank you very much.

The most grateful considerations for my wife, Cecy for the time, ideas and believes that coincide in the same place.

Sincerely,

Patricio Ponce-Barajas

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