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).

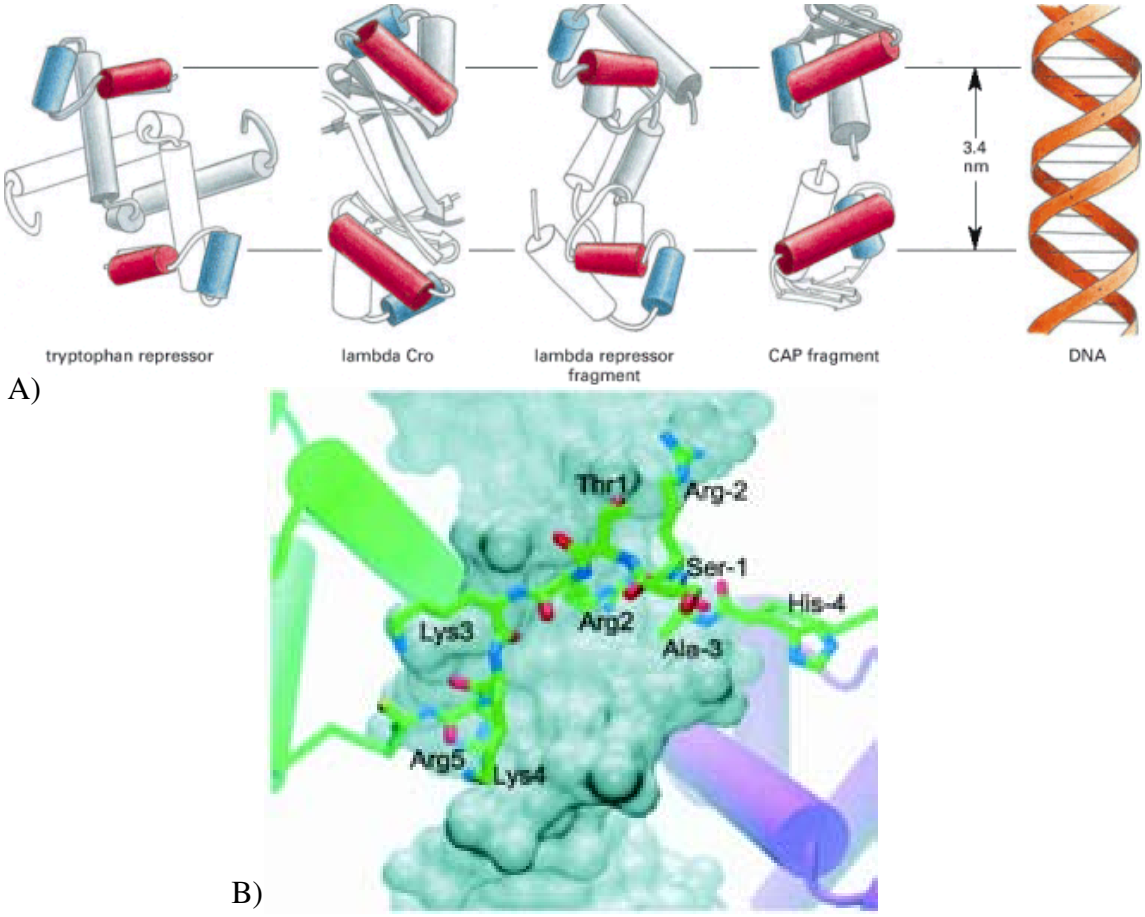

Figure 2.1 A) Example of some helix-turn-helix DNA-binding proteins of *E. coli* genes. These proteins bind DNA has dimers in which the two copies of the recognition helix (red cylinder) are separated by exactly one turn of the DNA helix, the external helix is coloured in blue. The helix-turn-helix motif 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