Developmental Genetics 15

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Introduction

Recent progress in developmental genetics has been dramatic. This includes extensive studies of mammalian development. However, the vast majority of data concerning mammalian development were generated by murine genetics. This means that, in discussing the genetic aspects of development in cattle, we cannot avoid significant gaps and have to refer to other species, mainly to the mouse.

Despite the high level of similarity in mammalian development, there are numerous contrasts between species, resulting from their morphological differences, placental structure, longevity and schedule of development. These distinctive features of development are certainly based on genetic differences, many of which are still awaiting clarification. Thus, the main objective of this chapter is to collect and pull together all available and relevant data concerning genetic determination of cattle development, and to supplement these data with information from other species in order to achieve better coverage of the topic. It is our hope that future reviews in this area will be able to promote significantly knowledge in developmental genetics of cattle using this initial set of data.

Developmental Stages of the Cattle Embryo

Gamete maturation and fertilization, which comprise the first and very fateful part of each new developmental cycle in mammals, have been considered in the previous chapters. The embryological events and their genetic determination which follow after fertilization are discussed here. Table 15.1 summarizes essential events and the timing of conceptus and fetal development in cattle. Accordingly, three consecutive periods are emphasized: ovum, embryonic and fetal.

The ovum (or preimplantation) period covers the first 15–16 days after fertilization and results in a mature blastocyst ready to implant. This period is characterized by several crucial events, including cleavage, morula formation and compaction, cavitation and blastocyst development. The latter includes hatching (release) from the zona pellucida, blastocyst elongation, gastrulation, somite formation and trophoblast development. The ovum stage in cattle and sheep is longer than in some studied mammalian species (Calarco and McLaren, 1976; Ménézo and Renard, 1993). For the first 5 days, seven cycles of cell division take place and the morula appears. Several hours later, compaction of the morula brings the developing embryo to the next stage – the blastocyst. Tight intercellular junctions develop and these provide a condition

Table 15.1. Essential events and timing of cattle prenatal development (compiled from: Winters *et al.*, 1942; Cruz and Pedersen, 1991; Bazer *et al.*, 1993; Guillomot *et al.*, 1993; Jainudeen and Hafez, 1993; Ménézo and Renard, 1993).

Stage of development	Days after fertilization	Cells/stage
Ovum period	0–16	
Cleavage		
Two-cell	1	2
Eight-cell	2–3	8
Genome activation	3–4	8–16
Entry into the uterus	4	16–32
Morula compaction	5–6	64–128
Blastocyst formation	6–7	128
Hatching	9–10	~200
Blastocyst elongation	11–16	~1000
Trophoblast differentiation	12–18	Thousands
Gastrulation	14–16	Thousands
Notochord	15–16	Thousands
Open neural tube	15–17	Thousands
Differentiation of the first somite	16–17	Thousands
Embryonic period	16–45	
Maternal recognition of pregnancy	16–19	Early somites
Implantation	18–20	Early somites
Head fold	19–20	Somites
Initial placentation	22	Somites
Closed neural tube	22–23	18–19 somites
Optic and otic vesicles, heart	22–23	18–19 somites
Gastrointestinal structures and mesonephros	26	Later somites
Visible forelimb and hindlimb buds	27–28	Vertebrae develop
Fetal period	46–parturition	
Eyelids close	54–59	Growing fetus
More developed form of head and neck	55–60	Growing fetus
Further development of limbs	60–70	Growing fetus
Hair follicles	90 and later	Growing fetus
Horn pits	100	Growing fetus
Hair coat	230	Growing fetus
Birth	276–290 (depending on the breed)	

for the accumulation of fluid within the central cavity (the blastocoele). The majority of cells in the blastocyst, called trophoblast or trophoectoderm, create a layer, which later becomes the chorion and has important trophic functions. The small group of cells located at one pole beneath the trophoblast forms the embryoblast or inner cell mass (ICM). Later, during gastrulation, the ICM differentiates into the three primary germ layers of the embryo (ectoderm, mesoderm and endoderm). During the same time, starting from day 12 of gestation, the trophoblast develops significantly. Further development of the conceptus assembles the essential conditions for implantation. These include the development of the embryo *per se* (notochord and neural tube formation, differentiation of the first somite, etc.), significant development of extra-embryonic tissues and development of maternal recognition of pregnancy.

Starting from this point (about 15–16 days after fertilization), the bovine embryo is entering into a new period of its development – the embryonic stages. The important features of this stage are embryo attachment to the

uterine wall and further development of extraembryonic structures and the placenta. This attachment in the cow is superficial and non-invasive (Winters *et al.*, 1942; Bazer *et al.*, 1993). Details are discussed later in this chapter in relation to freemartinism in cattle. During this period, the embryo develops all of its main organ systems and the shape of the embryo changes dramatically. Essential morphogenetic events, such as head, vertebrae and appendage formation, and the development of the nervous system, blood circulation and all other major internal organs occur during this time. By day 45 of gestation, the embryo develops well-recognizable features of the species.

The fetal period continues significantly longer than the two previous periods and covers the last 230–240 days of gestation, when the bovine fetus undergoes extensive growth and final development. Numerous morphological changes, although definite, are not radical. These changes are rather gradual and shape all fetal structures and functions towards requirements for postnatal life.

A comparison of developmental events and gene regulation during embryogenesis in cattle with other farm mammals can be fruitful (Cockett, 1997; Pomp and Geisert, 1998).

Genetic Control of Cleavage and Blastocyst Formation

Expression of maternal genes

The first three cleavage divisions in cattle occur mostly without activation of the embryo genome (Ménézo and Renard, 1993). This stage is covered by information, energy and structural molecules, mainly accumulated during oogenesis. The total ribonucleic acid (RNA) content in the zygote and in early blastomeres in mammals is commonly much higher than in somatic cells. The oocyte and the following early stages of development have the ability to perform polypeptide syntheses in the absence of active transcription. Experiments with a specific inhibitor of RNA polymerase II (α-amanitin) show that cleavage and probably polypeptide synthesis are not significantly affected until the four- to eight-cell embryo, but when added in later stages it completely inhibits further embryonic development (Barnes and First, 1991; Liu and Foote, 1997). Synthesis of heterogeneous RNA (hnRNA) is absent or very slight until the eight-cell embryo (Plante *et al.*, 1994; Viuff *et al.*, 1996; Lavoir *et al.*, 1997). Somatic histone H1, which supposedly regulates critical aspects of chromatin activity during early embryogenesis, assembles on embryonic chromatin during the fourth to sixth cell cycle after fertilization (Smith *et al.*, 1995).

Protein synthesis is not well pronounced until the 16-cell stage in bovine embryos, and embryonic cell ultrastructure supports biochemical observations. Nevertheless, some protein processing evidently does occur (Lavoir *et al.*, 1997). During this period, cellular mass decreases about 20%; however, nuclei increase in size (McLaren, 1974; Bazer *et al.*, 1993).

It is well known that in *Drosophila melanogaster* and *Caenorhabditis elegans* gradients of morphogens in the zygote and early embryo are crucial for establishing positional information (St Johnston and Nüsslein-Volhard, 1992; Nüsslein-Volhard, 1996). These gradients are essentially products of the expression of maternal genes. In what degree similar gradients and elements of cytoskeleton are important during the earliest stage of mammalian development remain to be seen, but it seems unlikely that these factors are not essential, at least in specifying major polarities (Holliday, 1990). Increasing cell polarity was described at the eight-cell stage of mouse and rat development (Reeve, 1981; Gueth-Hallonet and Maro, 1992). Cell fate, controlled by positional information, seems reversible and provides the developing embryo with a certain degree of flexibility. In cattle, cellular polarization occurred in some blastomeres at the nine- to 15-cell stage, but typical distinct polarity was not manifested until after the 16-cell stage, with approximately 40% polar cells per embryo (Koyama *et al.*, 1994).

There are data indicating a low gene expression in the mouse embryo during the first divisions (Davis and Schultz, 1997), but it is rather likely that the replication of deoxyribonucleic acid (DNA) and maintenance of the majority of cellular functions during the first two to three divisions are provided by RNAs and proteins accumulated during oocyte maturation. Oviduct proteins are also involved in this early stage of development. Oestrus-associated glycoprotein (EGP), for instance, is involved in modulation of the cleavage rate (Nancarrow and Hill, 1995).

Genome activation

The latest data suggest that the change from maternal to embryonic control starts as early as the two-cell stage in cattle (Memili *et al.*, 1998). Changes in nucleolus organizer regions (Ag-NORs) and in nucleolus ultrastructure occurring around the eight- to 16-cell stage in bovine embryos suggest a significant transcriptional activation of the ribosomal RNA (rRNA) genes and hnRNA production (King *et al.*; 1988, Lavoir *et al.*, 1997). This is clearly an important step in the activation of protein synthesis machinery. Cytogenetic investigations have also shown that on day 5 the developmental rates were slowest in haploid and polyploid embryos, intermediate in aneuploid embryos and fastest in mixoploid and diploid embryos (Kawarsky *et al.*, 1996). These data indicate active involvement of the embryonic genome at this stage, as well as the importance of diploid balance. In the mouse and probably in other mammalian species, the vast majority of more or less serious deviations from the standard chromosome set are not viable (Dyban and Baranov, 1988).

Physiological studies clearly show significant changes in the metabolic activity of the bovine embryos during early stages of development (Thompson *et al.*, 1996). For instance, adenosine triphosphate (ATP) production and oxygen consumption increase with compaction and blastulation. These data revealed that bovine embryos were dependent on oxidative phosphorylation

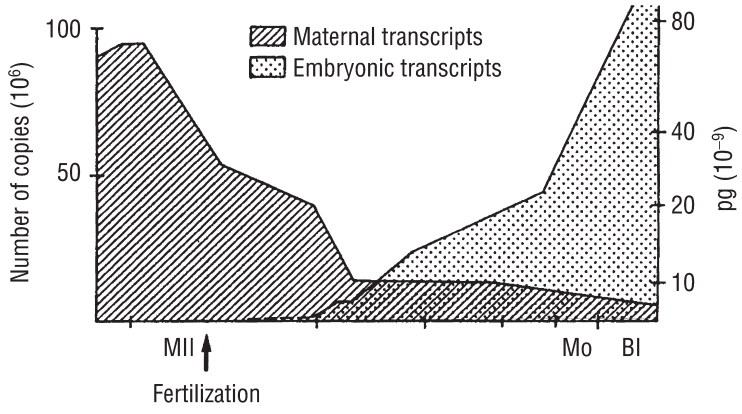

Fig. 15.1. Evolution of polyA$^+$ RNA during the preimplantation development of the mouse embryo (from Ménézo and Renard, 1993, with permission of Ellipses). MII, second meiotic metaphase; Mo, morula; Bl, blastula.

for energy production at all stages of pre-elongation development, with a shift in dependence towards glycolysis in conjunction with compaction. Figure 15.1 shows evolution of the polyA RNA profile during preimplantation development in the mouse embryo. A similar picture is expected for bovine embryos, except with a different time-scale. Expression of various developmentally regulated markers provides valuable information concerning early gene expression in bovine development. It was shown that lamin B appeared as a constitutive component of nuclei at all preimplantation stages but lamins A/C had a stage-related distribution (Shehu *et al.*, 1996). The nuclei from the early cleavage stages contained lamins A/C, which generally disappeared later, with a few possible exceptions in the morula and blastocyst. Several other proteins essential for morphogenetic events appear in developing bovine embryos. This includes several cytoskeletal and cytoskeleton-related components, such as F-actin, α-catenin and E-cadherin. These proteins appear starting from day 6 and their appearance or polarized distribution is relevant to morula compaction (Shehu *et al.*, 1996). Pig data suggest that several more morphogenetically important proteins appear during early cleavage and compaction, such as actin and actin-associated proteins, α-fodrin, vinculin and E-cadherin (Reima *et al.*, 1993). This is a result of activation of the corresponding genes and coincides well with early morphogenetic events. In the pig, these molecules are distributed evenly in blastomeres during early cleavage but are then gradually accumulated in regions of intercellular contacts towards the blastocyst stage (Reima *et al.*, 1993).

Embryonic gene expression

The blastocyst formation creates two totally different cell lineages: non-polarized inner mass cells and polarized trophectoderm or outer cells, having

microvilli. It was shown that in mice this process is accompanied by specific redistribution of the actin-associated protein ezrin, which has been proposed to play a role in the formation of microvillous structures (Louvet *et al.*, 1996). These microvilli play an early role in implantation (see section on implantation in this chapter). Before morula compaction, ezrin is located around the cell cortex. However, later, after blastocyst formation, it segregates to outer trophectoderm cells, with microvilli. Two phosphorylated forms of ezrin are present from the ovum period throughout preimplantation development, but the amount continuously decreases. A third non-tyrosine-phosphorylated isoform appears at the eight-cell stage and increases to blastocyst stage (Louvet *et al.*, 1996); the activation of a specific gene would be a reason for this. Several other actin-associated proteins mentioned above (α-fodrin, vinculin and E-cadherin) are involved in cytokeratin bundles, which are not observed until the early blastocyst in both the mouse and the pig (Reima *et al.*, 1993). It was found that the E-cadherin cell-adhesion function is essential in the establishment and maintenance of epithelial-cell morphology during embryogenesis and adulthood. Mouse embryos homozygous for a targeted mutation show severe abnormalities before implantation, because dissociation of adhesive cells of the morula occurs shortly after compaction and their morphological polarization is then destroyed. Maternal E-cadherin is able to initiate compaction, but cannot maintain the process (Riethmacher *et al.*, 1995). Significant defects in the cell-junctional and cytoskeletal organization were found in E-cahedrin-negative mouse embryos (Oshugi *et al.*, 1997).

In spite of the differences found in the mouse and the pig in the timing of events, in both species a close correlation between developmental stage and the organization of the cytoskeleton was observed. In the expanded bovine blastocyst, the distribution of cytoskeletal and cytoskeleton-related proteins looks similar (Shehu *et al.*, 1996). Extracellular fibronectin was first detected in the early blastocyst before differentiation of the primitive endoderm, and at this stage it was localized at the interface between the trophectoderm and extraembryonic endoderm (Shehu *et al.*, 1996). A connection between switching on particular genes, accumulation of proteins and morphogenetic events is visible. Cingulin, the tight-junction peripheral-membrane protein, also contributes to morphological differentiation in early development, and is found in mice. It is likely that other mammals have the same gene. Its synthesis is tissue-specific in blastocysts and is upregulated in the trophectoderm and downregulated in the ICM (Javed *et al.*, 1993).

It is commonly accepted now that proto-oncogenes are deeply involved in numerous processes of embryonic development determining nuclear transcription factors, intracellular signal transducers, growth factors and growth-factor receptors. For instance, activation of the *c-fos* and *c-jun* proto-oncogenes in sheep conceptuses occurs during the period of rapid growth and elongation (Wu, 1996). A similar pattern could be typical for bovine embryos in the time around implantation. These proto-oncogenes are involved in the regulation of gene expression, cell proliferation and differentiation.

To estimate the dynamics of developmental activity of some housekeeping genes in *in vitro* bovine embryos, the following gene products were studied: two mitochondrial transcripts, 12S rRNA and cytochrome b messenger RNA (mRNA); two RNAs involved in the processing of other RNAs, U2 and U3 snRNA; and two nuclear-derived transcripts, β-actin mRNA and histone H3 mRNA (Bilodeau-Goeseels and Schultz, 1997). The RNA levels for the various genes studied remained constant or decreased slightly from the mature oocyte to the six- to eight-cell or morula stage and greatly increased in blastocysts. Increases in gene expression were significant, ranging from two- to sixfold to 110–118-fold.

It was found that at least some regulatory substances secreted by the uterus can act as growth factors. Together with a number of growth factors and their receptors produced by the embryo, they create the medium essential for development. A detailed review of these regulators of mammalian embryonic development is published elsewhere (Schultz and Heyner, 1993). In early bovine embryos, transcripts for insulin-like growth factors (IGF-I, IGF-II) and mRNAs encoding receptors for insulin were detectable at all embryo stages, including the blastocyst. It is suggested that these transcripts are products of both the maternal and embryonic genomes (Schultz *et al.*, 1992). Genes for insulin-like growth factors (*IGF*s), transforming growth factors (*TGF*s), fibroblast growth factor (*FGF*), platelet-derived growth factor (*PDGF*) and also receptors for insulin, *IGF*, *PDGF*, *TGF* α and epidermal growth factor (*EGF*) are expressed by early bovine embryos (Harvey *et al.*, 1995). Harvey and co-workers (1995) reported that successful development of the blastocyst is dependent on the action of EGF and leukaemia inhibitory factor (LIF). The latter may act directly or indirectly, by inducing the expression of other cytokines, to regulate the temporal and spatial production of proteases and protease inhibitors, and to create conditions for implantation at least in the mouse (Harvey *et al.*, 1995).

Many efforts have been contributed toward answering the question whether development of preimplantation embryos depends on internal and/or external factors. Most experiments with rodents show that it is unlikely that preimplantation development is significantly dependent on exogenous factors. Furthermore, none of the known endogenously produced factors and their receptors are essential during preimplantation development to the blastocyst stage (Stewart and Cullinan, 1997). However, later during development, the importance of growth factors rises sharply.

Trophoblast gene expression

Differentiation of trophoblast cells, the first and perhaps the most radical event during mammalian embryonic development, provides an embryonic component for the future fetal–maternal interface during implantation and placentation. A detailed description of current knowledge about the genetic control of trophoblast development and implantation is presented in a special

issue of *Developmental Genetics* (Schultz and Edwards, 1997). Clearly, many features of these processes are common for the majority of eutherian mammals and applicable to cattle. At the moment, 44 loci with different functions implicated in preimplantation or peri-implantation events have been identified (Rinkenberger *et al.*, 1997).

A basic helix–loop–helix (bHLH) transcription factor gene, *Hxt*, is expressed in the early trophoblast and in differentiated giant cells of mouse embryos (Cross *et al.*, 1995). The negative HLH regulator, *Id-1*, inhibited rat trophoblast (Rcho-1) differentiation and placental lactogen-I transcription. These data demonstrate a role for HLH factors in regulating trophoblast development, at least in rodents, and indicate a positive role for *Hxt* in promoting the formation of trophoblast giant cells. A separate gene, *Hed*, encodes a related protein, which is expressed in maternal decidium surrounding the implantation site (Cross *et al.*, 1995). One of them is *Mash-2*, a locus homologous to the *Drosophila achaete/scute* complex genes, and this determines the transcription factor. Its expression begins during preimplantation development, but is restricted to trophoblast lineage after the blastocyst stage (Nakayama *et al.*, 1997). This murine locus belongs to the quite rare category of imprinted genes (Guillemot *et al.*, 1995). Mouse embryos that inherit a mutant allele from the mother and normal from the father die after implantation. The cause of death is a lack of placental spongiotrophoblast (McLaughlin *et al.*, 1996). The *MMp9* gene, which is involved in development of giant trophoblast cells in mice, is one more candidate for imprinted genes (Newman-Smith and Werb, 1997). Several more genes are currently under investigation to determine their role in trophoblast development and implantation/placentation events (Schultz and Edwards, 1997).

Genetic determination of integrin trafficking, which regulates adhesion to fibronectin during the differentiation of the mouse peri-implantation blastocyst, has been studied by Schultz *et al.* (1997). The regulation of several metalloproteinase and corresponding genes may also shed additional light on the process of implantation and further trophoblast development (Bass *et al.*, 1997; Das *et al.*, 1997). Because placental development in rodents is very different from that in cattle, further research will be needed to determine if these murine trophoblast genes are relevant to bovine trophoblast development.

Gametic imprinting

Gametic or genomic imprinting is a developmental phenomenon based on differential expression of maternal and paternal alleles in some genes typical for eutherian mammals. Until now, the main bulk of information regarding imprinting comes from the mouse and, to a lesser extent, from humans (Barlow, 1995). The number of imprinted genes known for the mouse is about 20. The genes are essential for regulation of embryonic and placental growth. These include paternally expressed genes – *Igf2*, *Snrpn*, *Ins*, *Znf127* – and maternally expressed genes – *Wt1*, *H19*, *Igf2r*. Imprint acquisition is believed

to occur before fertilization and imprint propagation takes place until the morula–blastocyst stage (Ruvinsky and Agulnik, 1990; Shemer *et al.*, 1996). The molecular mechanisms of gametic imprinting are still under intensive investigation. It seems likely, however, that primary gametic signals are not simply copied from the gametes, but rather that a methylation pattern typical for imprinted genes is gradually established during early development (Shemer *et al.*, 1996; Surani, 1998). The developmental function of gametic imprinting is also not absolutely clear, but an explanation proposed by Moore and Haig (1991) is widely accepted. It is based on the idea of genetic conflict arising during pregnancy between maternally and paternally inherited genes. Thus, it is likely that gametic imprinting evolved in mammals to regulate intrauterine growth and to increase safeness of embryonic development. Data on farm animals are still limited and include some indirect and direct evidence of gametic imprinting (Ruvinsky, 1999). In sheep the callipyge gene responsible for pronounced muscle hypertrophy in hindquarters is controlled by a novel form of imprinting, referred to as polar overdominance (Cockett, 1997). The latest experiments confirmed the existence of gametic imprinting in ruminants (Feil *et al.*, 1998).

Lack of maternally or paternally derived alleles in a zygote causes embryonic mortality in several instances and should impose strict requirements on the stability of imprinting signals. A recent report of cloning using somatic cells of adult sheep (Wilmut *et al.*, 1997) is the first indication that differential imprinting signals are maintained in somatic cells long after intrauterine development and are very stable.

It seems possible that knowledge about the influence of the pathway (paternal or maternal) used by an allele to enter the next generation will sooner rather than later be adopted by selection programmes. Selection of modifier genes may significantly change the effect of gametic imprinting and this knowledge should also be taken into consideration.

Implantation and Maternal Recognition of Pregnancy

Implantation

Placental attachment in cattle initially (between week 2 and 3 of pregnancy; see Table 15.1) involves minute papillae of embryonic membranes, which penetrate the vestibule of the uterine glands. These papillae disappear before day 30 of pregnancy (Guillomot and Guay, 1982) and are replaced by interdigitating microvillus connections of the placental allantochorion to maternal caruncular crypts of the uterine endometrium (King *et al.*, 1979). These placenta microvilli grow and become vascularized, as do the associated caruncular areas, which leads to the formation of placentomes, which take on a convex characteristic in cattle. Also, no syncytia form in the intercaruncular epithelium, but abundant giant cells are present during the fourth week of pregnancy and decrease in numbers thereafter (King *et al.*, 1981). Thus

implantation leads eventually to formation of extraembryonic membranes and placenta, which are vital for normal embryonic growth and development. Until recently, knowledge about genes involved in implantation was limited and this is still true for cattle. However, in the mouse significant progress has been achieved (Rinkenberger *et al.*, 1997). At least 40 different genes were identified, whose activity is necessary for normal development. A lack of any of them causes peri-implantation or placentation failure. The proposed or known functions of these genes are very diverse, including transcription factors, hormone receptors, cytokines and receptors, DNA-serving enzymes, adhesion molecules, etc. This breakthrough opens opportunities for similar investigations in cattle.

Cytokines in maternal recognition of pregnancy

In order to maintain a successful pregnancy in domestic mammals, the process of luteolysis has to be inhibited, as well as the immune response from the mother to foreign tissue in the uterus (i.e. the embryo). The conceptus is thought to be responsible for this signalling during its first few weeks of life (Roberts *et al.*, 1992; Bazer *et al.*, 1994; Thatcher *et al.*, 1995; Godkin *et al.*, 1997; Martal *et al.*, 1997). Conceptus signalling may be accomplished through the synthesis and secretion of an interferon (IFN)-like polypeptide known as trophoblast protein 1 (TP-1; Roberts *et al.*, 1992; Bazer *et al.*, 1994; Martal *et al.*, 1997), now termed IFN-τ. This protein has been identified in sheep (oTP-1), bovine (bTP-1) and caprine conceptuses (Stewart *et al.*, 1989; Geisert *et al.*, 1992; Bazer *et al.*, 1994). The conceptus's IFN-like protein, which is similar to bovine IFN-αII, has antiviral activity, and is effective against many viruses. Because of their structural resemblance, oTP-1 and IFN-αII compete for the same receptor (Stewart *et al.*, 1989; Roberts *et al.*, 1992). Numerous other cytokines, including interleukin (IL)-1, 2, 4, 6 and 10, colony-stimulating factor and tumour necrosis factor α (TNFα), have been implicated in mediating communications between the conceptus and maternal uterine epithelium, both before implantation and as the placenta develops (Robertson *et al.*, 1994).

Interferon τ prevents luteolysis through the inhibition of endometrial prostaglandin F2α (PGF2α) release in response to oestrogen and oxytocin triggering, and by suppressing inositol phosphate/diacylglycerol second-messenger pathways for PGF2α synthesis (Roberts *et al.*, 1992; Bazer *et al.*, 1994; Godkin *et al.*, 1997). Oestradiol and oxytocin are necessary mediators for the release of PGF2α (Geisert *et al.*, 1992; Bazer *et al.*, 1994; Godkin *et al.*, 1997) required for the initiation of luteolysis. Non-pregnant ewes that have been exposed to uterine infusion of oTP-1 between days 12 and 18 of an oestrous cycle have extended interoestrous intervals through maintenance of the corpus luteum (Vallet *et al.*, 1988). During maternal recognition of pregnancy, ovarian follicular populations are altered in cattle (Thatcher *et al.*, 1989; Spicer and Geisert, 1992). Thus, cytokines may play a role in the suppression of follicular development during this early stage of pregnancy by reducing the secretion of

oestradiol, which could otherwise stimulate uterine secretion of PGF2α (Spicer and Geisert, 1992; King and Thatcher, 1993). In support of these suggestions, exogenous IFN-α reduced progesterone concentrations in the blood of heifers (Newton *et al.*, 1990) and *in vitro* studies showed similar negative effects of IFNs on the steroidogenesis of bovine luteal cells (Fairchild and Pate, 1991) and bovine granulosa cells (Alpizar and Spicer, 1994). Furthermore, TNFα but not IL-1β inhibited luteinizing hormone (LH)-stimulated progesterone production by bovine luteal cells (Nothnick and Pate, 1990; Fairchild and Pate, 1991; Benyo and Pate, 1992), and IFN receptors have been identified in ovine corpora lutea (Godkin *et al.*, 1984). Collectively, these results indicate that cytokines have inhibitory effects on follicular and luteal steroidogenesis in cattle. Although TNFα is localized in bovine corpora lutea (Roby and Terranova, 1989) and receptors for TNFα are present in bovine granulosa and thecal cells (Spicer, 1998), as well as in porcine corpora lutea (Richards and Almond, 1994), the presence of factors that regulate TNFα receptors during luteal development in cattle has not been identified to date. Whether these and other cytokines are involved in the maternal recognition of pregnancy remains to be elucidated.

Placental gene expression

As mentioned earlier in the section 'Genetic Control of Cleavage and Blastocyst Formation', the limited evidence in bovine embryos indicates that the embryonic genome is not activated until the eight- to 16-cell stage (Camous *et al.*, 1986; Kopecny *et al.*, 1989; Kopecny and Fakan, 1992). Initiation of embryonic transcription during the eight- to 16-cell stage was further confirmed by studies using bovine embryos produced from *in vitro* matured and fertilized oocytes (Barnes and First, 1991; Rieger *et al.*, 1992). Recent evidence indicates that transcriptional regulation of a small number of specific genes in bovine embryos may occur as early as the four-cell stage (Edwards *et al.*, 1997). Specific genes expressed during preimplantation development were also described earlier (see the section 'Genetic Control of Cleavage and Blastocyst Formation'). Cytokine-related genes, such as bTP-1, are expressed as early as day 18 in bovine conceptuses (Cross and Roberts, 1991), whereas colony-stimulating factor receptor gene expression begins around day 29 in post-attachment bovine trophoblasts (Beauchamp and Croy, 1991).

Bovine placentae express numerous proteins and corresponding mRNAs during development, including prolactin-related protein I, II, III and IV (Schuler and Hurley, 1987; Kessler *et al.*, 1989; Milosavljevic *et al.*, 1989; Yamakawa *et al.*, 1990; Zieler *et al.*, 1990; Tanaka *et al.*, 1991; Anthony *et al.*, 1995; Kessler and Schuler, 1997), placental lactogen (Schuler *et al.*, 1988), prolactin receptor (Schuler *et al.*, 1997), aromatase (Hinshelwood *et al.*, 1995; Fürbass *et al.*, 1997), steroidogenic acute regulatory protein (StAR; Pescador *et al.*, 1996; Pilon *et al.*, 1997), procollagen III (Shang *et al.*, 1997) and an aspartic proteinase, bovine pregnancy-associated glycoprotein 1 (bPAG1; Xie *et al.*,

1994, 1995). The prolactin-related proteins are expressed predominantly in full-term bovine placentae (Yamakawa *et al.*, 1990; Tanaka *et al.*, 1991) and their genes have been assigned to bovine chromosome 21 (Dietz *et al.*, 1992), whereas procollagen III gene expression is detected in the bovine chorioallantois by day 20 of gestation and increases through day 36 (Shang *et al.*, 1997). The latter observation suggests that procollagen III may be involved in the development of the allantois. The temporal pattern of placental expression of placental lactogen, prolactin receptor, aromatase, StAR and bPAG1 has not been characterized in cattle to date.

Genes Involved in Control of Morphogenesis

Gastrulation

The development of the bovine epiblast starting from gastrulation and onwards requires further investigations. The data are very limited, particularly with respect to gene expression activity. It is known that bovine blastocysts hatch at 9–10 days (1.6 mm) and enlarge rapidly thereafter: 3.75 mm on days 11–12, about 10 mm on days 12–14. By day 16, the blastocyst has transformed into an attenuated, bilaminar blastodermic vesicle.

Gastrulation starts about this time, i.e. approximately 3–4 days before implantation (Cruz and Pedersen, 1991). Cell proliferation and rearrangement in the germinal disc are the main events during gastrulation in eutherian embryos. The most obvious feature of commencing gastrulation is the formation of the primitive streak. 'This process begins with the production and proliferation of mesodermal progenitor cells at the proximal (allantoic) end of the primitive streak; this position marks the future caudal end of the fetus. As ectodermal cells migrate through the primitive streak, they move both laterally and distally towards future cranial end of the embryo, extending the primitive streak towards the distal lip' (Wilkins, 1993).

The genes that are responsible for gastrulation in mammals are mainly unknown. The same is true for establishing anterior–posterior orientation. Two genes, the homoeobox gene *goosecoid* (*gsc*) and the winged-helix gene *Hepatic Nuclear Factor-3beta* (*HNF-3beta*) are co-expressed in all three germ layers in the anterior primitive streak and at the rostral end of mouse embryos during gastrulation (Filosa *et al.*, 1997). A member of the *FGF* family, *Ggf-4*, shows expression restriction to the primitive streak and expresses sequentially in developmental pathways such as mesoderm formation and myogenesis, playing a role in specific epithelial–mesenchymal interactions (Niswander and Martin, 1992).

The recently discovered murine *Axin* gene seems to be a crucial regulator in embryonic axis formation in vertebrates. This gene inhibits the so-called Wnt signalling pathway, arranged from several polypeptides and enzymes (Zeng *et al.*, 1997).

The *T* gene, which is required for extension of the posterior axis (Clements *et al.*, 1996) and for several other essential steps in mammalian development, including notochord formation, is discussed below. In the mouse and other mammals, the next step of development is the so-called 'head process', which gives rise to the notochord and contributes to part of the endodermal lining of the gut.

Notochord formation

The notochord is a rod-shaped structure that extends along the embryo and represents the initial axial skeleton, playing an important role in the induction of the neural plate, chondrogenesis and somite formation (Gomercic *et al.*, 1991). Notochord development in the bovine embryo commences 15–16 days after fertilization (a couple of days before implantation), which is different from the mouse and human. It was found that the bovine notochord begins decomposing at the end of the embryonal and the start of the fetal period (45–50 days). After 55–65 days, it no longer presents an unbroken cord of notochord cells. The activity of several oxidative mitochondrial enzymes increases significantly from the early stage of notochord development (Gomercic *et al.*, 1991). Clearly, an activation of some nuclear genes responsible for basic morphogenetic rearrangements is required for notochord formation and development. The *T* gene, which was first described as *Brachyury* mutation in mice 70 years ago, is a very important participant in events required for differentiation of the notochord and the formation of mesoderm during posterior development. The T protein is located in cell nuclei and acts as a tissue-specific transcription factor (Kispert *et al.*, 1995). The cloning and sequencing of the *T* gene led to the discovery of the T-box gene family, which is characterized by a conserved sequence, called the T-box (Bollag *et al.*, 1994). This ancient family of transcription factors underwent duplication around 400 million years ago and is common to vertebrates (Ruvinsky and Silver, 1997). There are indications that several mouse T-box genes play an important role in different mesodermal subpopulations and one in early endoderm during gastrulation (Papaioannou, 1997).

Formation of the notochord leads to several very important events, including induction of the neural tube and development of the gut, heart and brain. A putative morphogen secreted by the floor plate and notochord, sonic hedgehog (Shh), specifies the fate of multiple cell types in the ventral aspect of the vertebrate nervous system. In turn, Shh induces expression of oncogene *Gli-1*, which affects the later development of the dorsal midbrain and hindbrain (Hynes *et al.*, 1997).

Somitogenesis proceeds soon after this (16–17 days) in the opposite, posterior, direction. At this stage (18–20 days), the bovine embryo begins implanting into the uterine wall (see the section 'Implantation and Maternal Recognition of Pregnancy').

Hox *genes and development of axial identity*

The homoeotic genes were first described in *Drosophila* as the primary determinants of segment identity, and they encode transcription factors. These genes share a very similar 180-bp DNA sequence, named the homoeobox. Comparative analysis of the *Drosophila* homoeotic gene complex, called *HOM-C*, and the mammalian homoeobox genes, called the *Hox* complex, showed a very striking case of evolutionary conservation. The *Hox* gene family determines a set of transcription factors crucial for development of axial identity in a very wide range of animal species (Maconochie *et al.*, 1996). Figure 15.2 shows the surprising similarity and collinearity found in the molecular anatomy of the insect and mammalian (vertebrate) complexes. The main difference is the number of the complexes per genome. In insects there is only one, while mammals and other vertebrates have four paralogous sets of genes.

There are no direct data about the structure and function of the bovine *Hox* complex, but it is very likely that the main features of the murine *Hox* complex are typical for it. The *Hox* genes are expressed in segmental fashion in developing somites and the central nervous system. Each *Hox* gene acts from a particular anterior limit in the posterior direction. The anterior and posterior limits are different for different *Hox* genes (Fig. 15.2). The genes located at the 3′ end have the most anterior limit of activity. The transcription of the genes, however, moves in the usual 5′ to 3′ direction. The genes located at the 3′ end express earlier and genes located at the 5′ end work later. The process of segmentation moves along the anterior–posterior (A–P) axis. There are differences in the development of segmentation between the hindbrain and the trunk (Maconochie *et al.*, 1996). Thus, the vertebrate body is at least partially a result of interactions of *Hox* genes, which provide cells with the essential positional and functional information they require to migrate to an appropriate destination and generate the necessary structures. Retinoids can affect the expression of *Hox* genes and there is a 5′ to 3′ gradient in responsiveness of *Hox* genes to retinoids, based on the response elements of some *Hox* genes (Marshall *et al.*, 1996).

A key role of the neural crest as the source for numerous cell lineages, including sensory neurons, glia cells, melanocytes, some bone and cartilage cells, thyroid cells and smooth muscle, is well known (Le Douarin, 1982). Progress during the past few years has been significant in the identification of the genes controlling the development of the neural crest and cell migration (Anderson, 1997). Several growth factors affect the developmental fate of neural-crest cells: the glial growth factor (GGF), TGF-β (promoting smooth-muscle differentiation) and BMP2/4 (involved in bone morphogenesis). Transcription factors are also important in neural-crest lineage determination, among them bHLH transcription factors. The genes *Mash1* and *Mash2* are responsible for the production of these factors (Anderson, 1997).

Organogenesis: T-box, Pax *and other genes*

Some of T-box gene family are involved in limb morphogenesis and specification of forelimb/hindlimb identity. It has been shown that *Tbx5* and *Tbx4* expression is primarily restricted to developing the fore- and hindlimb buds,

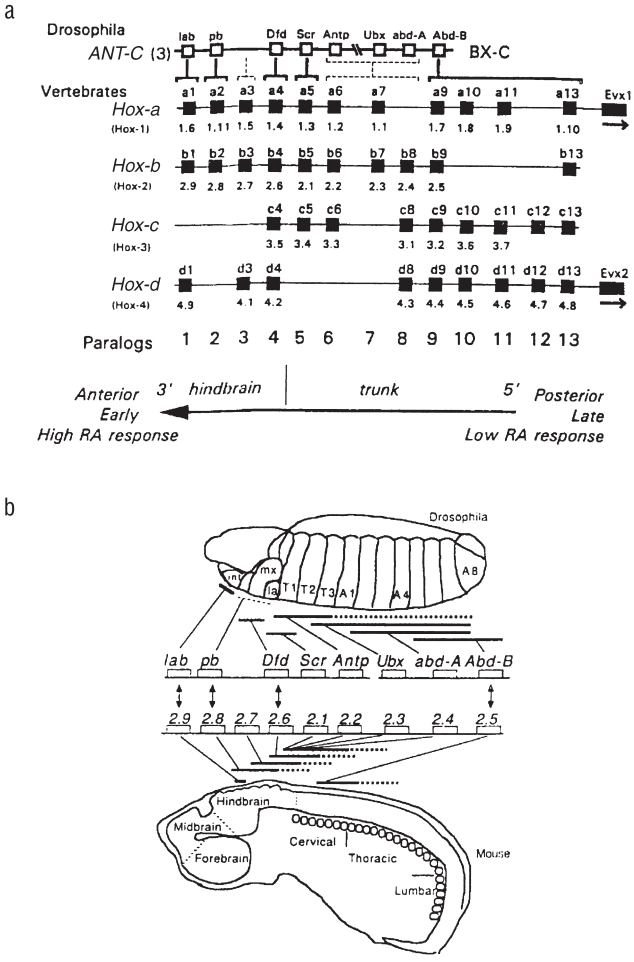

Fig. 15.2. (a) Alignment of the four mouse *Hox* complexes with that of *HOM-C* from *Drosophila*. The vertical shaded boxes indicate related genes. The 13 paralogous groups are noted at the bottom of the alignment. The colinear properties of the *Hox* complexes with respect to timing of expression, anteroposterior level and retinoic acid (RA) response are also noted at the bottom. (From Maconochie *et al.*, 1996, with the authors' permission.) (b) Summary of *HOM-C* and *Hox-2* expression patterns. The upper part of the figure is a diagram of a 10-h *Drosophila* embryo with projections of expression patterns of different genes from *HOM-C* complex to particular body segments. The lower part of the figure is a diagram of a 12-day mouse embryo with projections of expression patterns of different genes from the *Hox-2* complex to particular body segments. (From McGinnis and Krumlauf, 1992; with the authors' permission).

respectively. These two genes have probably been divergently selected in vertebrate evolution to play a role in the differential specification of fore- (pectoral) versus hind- (pelvic) limb identity (Gibson-Brown *et al.*, 1996). Mutations in human TBX3 cause the ulnar–mammary syndrome, characterized by posterior limb deficiencies or duplications, mammary gland dysfunction and genital abnormalities. It was suggested that *TBX3* and *TBX5* evolved from a common ancestral gene and each has acquired specific, yet complementary, roles in patterning the mammalian upper limb (Bamshad *et al.*, 1997).

Pax genes are another family of developmental genes, coding nuclear transcription factors. They contain the paired domain, a conserved amino acid motif with DNA-binding activity. *Pax* genes are key regulators in organogenesis of some tissues and organs, including the kidney, eye, ear, nose, limb muscles, vertebral column and brain. Vertebrate *Pax* genes are involved in pattern formation, possibly by determining the time and place of organ initiation or morphogenesis (Dahl *et al.*, 1997). *Pax-1*, for instance, is a mediator of notochord signals during the dorsoventral specification of vertebrae (Koseki *et al.*, 1993). *Pax-3* may mediate the activation of *MyoD* and *Myf-5*, the myogenic regulatory factors, in response to muscle-inducing signals from either axial tissues or overlying ectoderm, and may act as a regulator of somitic myogenesis (Maroto *et al.*, 1997). Mutations in *Pax-6* result in eye malformation, known as Aniridia in humans and small eye syndrome in mice, demonstrating involvement of this gene in eye formation; the renal coloboma syndrome is a result of mutation in *Pax-2* (Dahl *et al.*, 1997). Another eyes-absent gene (*Eya2*) is involved in eye development in several metazoan phyla and should also play an important role in cattle. Like the *Pax-6* gene family, *Eya2* was probably recruited for visual system formation significantly later than its occurrence (Duncan *et al.*, 1997). Several more genes, such as *Bmp-4*, *Msx-1* and *Msx-2*, coding bone-morphogenetic proteins, which are expressed before neural-tube closure and later on, interact with *Pax-2* and *Pax-3* (Monsoro-Burq *et al.*, 1996).

The study of the dynamics of gene expression and regulation in the bovine photoreceptor system, including rhodopsin, arrestin, the rod alpha-subunit of transduction, interphotoreceptor retinoid-binding protein and the rod alpha-subunit of cyclic guanosine monophosphate (cGMP) phosphodiesterase (Timmers *et al.*, 1993), as well as opsin (DesJardin *et al.*, 1995), shows a complicated picture of gene activity changes and interactions during the final maturation of the system.

Muscle development and gene regulation

The development of muscle tissue is of particular interest for cattle, as this can provide important information from a practical point of view. For instance, it was shown that fetal muscle development differs in cattle that have different postnatal growth patterns by as early as 100 days of gestation, and this is a muscle hyperplasia-dependent phenomenon (Gore *et al.*, 1994). The most

recent progress on genetic mechanisms of muscle development was reviewed by Firulli and Olson (1997). Skeletal, cardiac and smooth-muscle cells express overlapping sets of muscle-specific genes; however, there are genes expressed in one particular muscle type. So-called modules or independent *cis*-regulatory regions are required to direct the complete developmental pattern of expression of individual muscle-specific genes, even within a single muscle-cell type. The temporospatial specificity of these myogenic regulatory modules is established by unique combinations of transcription factors (Firulli and Olson, 1997).

At least two generations of cells during fetal development in cattle were observed. One appears at a very early stage and gives rise to adult fast type I fibres. A second generation of cells gives rise to adult fast type IIA and IIB fibres and to type IIC. The beginning of myogenesis is characterized by expression of transitory myosin forms that are not found in adult cattle (Picard *et al.*, 1994). Insulin-like growth factor II modulates myogenesis and, between 60 and 162 days of gestation, the majority of IGF-II was localized to developing muscle cells. Probably, IGF-II acts as an autocrine-acting growth factor during myogenesis (Listrat *et al.*, 1994). Bovine cathepsin B, a lysosomal cysteine proteinase, is involved in fetal muscle development and is encoded by two different transcripts resulting from alternative polyadenylation. These two mRNAs declined similarly from 80 to 250 days of fetal age (Bechet *et al.*, 1996). The bovine fetal skeletal muscle myosin heavy-chain gene expression is influenced by genotype and indirectly by external factors (Gore *et al.*, 1995).

Molecular and developmental studies of double-muscling in cattle caused by the recessive mutation *mh*, located at chromosome 2, shed additional light on skeletal-muscle development (Charlier *et al.*, 1995; Grobet *et al.*, 1997). It was shown that an 11-bp deletion in the coding sequence of the myostatin gene, which belongs to the *TGF*-beta superfamily, caused the muscular hypertrophy (double-muscled phenotype) in Belgian Blue (Grobet *et al.*, 1997; Kambadur *et al.*, 1997). In Piedmontese cattle, a G–A transition in the same region of the gene is responsible for the phenotype (Kambadur *et al.*, 1997). It is likely that myostatin is a negative regulator of muscle growth in cattle and other mammals. It was also found that differentiation of the muscle fibres occurs at a slower rate in double-muscled fetuses, particularly during the first two-thirds of gestation (Picard *et al.*, 1995). Experiments where one fetus was double-muscled and the co-twin was normal show that genetically double-muscled bovine fetuses do not develop their muscles as might be expected. These data strongly support the idea that blood-borne factors regulate muscle hypertrophy in fetal cattle (Gerrard *et al.*, 1995).

A description of bovine fetal growth and development from the 90th to the 255th day of gestation is presented elsewhere (Prior and Laster, 1979). This includes numerous data concerning the dynamics of fetal weight, ash, protein, RNA and DNA content and other parameters. A more detailed study of fetal and placental growth in Herefords and crossbreds has been conducted by Anthony *et al.* (1986).

Developmental effects of coat-colour mutations

Classical coat-colour genetics in mammals has recently acquired a developmental and molecular orientation (Jackson, 1994). Most data were obtained within mouse genetics, but the high homology of mammalian genomes provides a sufficient foundation for extension to cattle (for more information, see Chapter 3).

There is a group of mutations that affect melanocyte differentiation, proliferation and migration and are responsible for development of a different kind of white spots. It was shown that neural-crest melanoblast migration is dependent on a signal encoded by *Steel* and a receptor encoded by the *W* locus in mice. A tyrosine-kinase receptor protein is a product of the proto-oncogene *Kit* (*W* locus), and its ligand, encoded by the *Steel* locus, is a membrane-bound protein. Mutations in one or both loci cause white spots and numerous neurological, haematological and fertility effects, which are in many cases lethal (Fleischman, 1993). The *roan* locus in cattle was mapped to the same interval on chromosome 5 as the candidate gene *Steel* (Charlier *et al.*, 1996). The dominant *R* allele plays the critical role in the determination of white heifer disease, which stems from its pleiotropic effect on fertility. Details are considered below in the paragraph devoted to sex differentiation.

Mutations that affect melanocyte morphology and create dilute colours are common in mammals. It was shown that mutations in a myosin protein (Jackson, 1994), which may be caused by proviral insertion (Jenkins *et al.*, 1981), lead to lack of dendrites by melanocytes and diluted coat colour. Interestingly, neuronal dendrites are not affected by these mutations.

Several other mutations affect melanogenic enzymes and related proteins. Tyrosinase gene mutations lead to albino variants. Brown colours, at least in mice, are the product of mutations in locus coding tyrosinase-related protein (Jackson, 1994). Pleiotropic effects, including decreased viability, are known.

Mutations of two other coat-colour loci, *agouti* and *extension*, affect the regulation of melanogenesis. It was shown that the ratio between black eumelanin and yellow phaeomelanin is regulated by the α-melanocyte-stimulating hormone (αMSH). The product of the *agouti* gene works as an antagonist of αMSH and the *extension* gene encodes the αMSH-receptor. Again, there are several developmental effects of these loci, including obesity and tumour formation, which attract further attention. Several more examples demonstrating connections between coat colour and adaptations in cattle are presented in Chapter 22. Black, brown and red coat colour in cattle are postulated to be produced by the E- and A-loci allele interactions (Adalsteinsson *et al.*, 1995) and corresponding mutations in the MSH receptor were observed (Klungland *et al.*, 1995). Red colour in Holstein cattle is indeed associated with a deletion in the MSHR gene (Joerg *et al.*, 1996).

Thus, molecular analysis of coat-colour mutations provides an excellent opportunity for a better understanding of some basic developmental processes.

Sex Determination and Differentiation

The major steps in gonad differentiation

It was known long ago that in mammals the sex determination mechanism is based on the presence or absence of the Y chromosome. Embryos without the Y chromosome develop as females and those with the Y chromosome develop as males. A recent breakthrough in the molecular understanding of sex determination and differentiation in the mouse and human (Goodfellow and Lovell-Badge, 1993) paved the way for other mammals, including cattle.

In humans and mice, gonadal differentiation starts relatively late in embryonic development and morphological differences in XY embryos appear prior to those in XX embryos. A similar situation is probably true for cattle sex differentiation. In cattle, androstenedione metabolism commences in male embryos at about 25–27 days of gestation and in female embryos several days later, and this occurs at the stage when gonads still look similar (Juarez-Oropeza *et al.*, 1995). The morphologically observable sex differentiation of ovaries takes place in bovine embryos in about 45-day-old female embryos (Kurilo *et al.*, 1987). It is suggested that differentiation of the gonads starts about a week earlier in males than in females. Entry of the oocytes into meiotic prophase occurs in 9-month-old fetuses.

It was shown that testicular development is a key element in establishing mammalian sex. However, it is noteworthy to mention that XY embryos show faster development during the first 8 days than XX embryos, long before the indifferent gonad even appears. These findings suggest that sex-related gene expression affects the development of embryos soon after activation of the embryonic genome (Xu *et al.*, 1992).

The chromosomal sex of germ cells determines their migration pattern and final differentiation into a testis or an ovary. Testicular development is triggered by a gene on the Y chromosome coding for a testis-determining factor (*SRY*). This factor induces, in genetic males, differentiation of Sertoli cells (reviewed by McLaren, 1991) secreting anti-Müllerian hormone (AMH). The latter belongs to the family of TGF-β, causes regression of the Müllerian ducts, promotes the development of Wolffian ducts and promotes differentiation of Leydig cells. The Leydig cells secrete the male steroid hormone, testosterone (Behringer, 1995). Testosterone binds to androgen receptors, which in turn act as transcription factors. More details about AMH and its activity in bovine development are presented elsewhere (Cate and Wilson, 1993). In bovine fetuses, the regression of Müllerian ducts occurs simultaneously in males and freemartins between 50 and 80 days of development (Vigier *et al.*, 1984). A whole chain of developmental events follows, and the phenotype typical for males arises. In females, Müllerian ducts develop, no Leydig cells form in the gonad, no testosterone is produced and development moves steadily towards the female phenotype. The female developmental programme is basic or 'default', while the male programme ultimately requires switching on the *SRY* gene.

The SRY *gene*

The testis-determining role of the *SRY* gene in mammals is widely accepted after impressive experiments (reviewed by Goodfellow and Lovell-Badge, 1993). The bovine *SRY* gene has been cloned and sequenced. The putative bovine SRY protein consists of 229 amino acid residues, with sequence conservation between species, notably in the region of the so-called high-mobility group (HMG) box (Daneau *et al.*, 1995). This conserved 79 amino acid motif confers DNA-binding ability and probably acts as a transcription factor (Goodfellow and Lovell-Badge, 1993). Apart from the HMG box, the bovine SRY structure shows greater resemblance to human *SRY* than to mouse *Sry* (Daneau *et al.*, 1995).

Expression of the *SRY* gene was confirmed in bovine embryos, both for a short time at the sex-determining stage of development around the period of the primitive undifferentiated gonad and in adult testes (Gutierrez-Adan *et al.*, 1997). Expression of SRY was also reported as early as the four- to eight-cell stage up to the blastocyst stage in bovine embryos. Expression of *SRY* at these early stages and the previous observation that *in vitro*-produced male bovine embryos develop faster in culture than female embryos suggest that sex differences are evident prior to gonadal differentiation and that preimplantation bovine embryos have sexually dimorphic gene expression at least with respect to *SRY* transcripts (Gutierrez-Adan *et al.*, 1997).

Several more genes have been implicated in the process of mammalian sex determination since the cloning of the *SRY* gene (Ramkissoon and Goodfellow, 1996). This set includes the *SRY*-related HMG box (*SOX*) gene family, which displays properties of both classical transcription factors and the architectural components of chromatin (Pevny and Lovell-Badge, 1997). *Sox9* has an essential function in sex determination, possibly downstream of *Sry* in mammals, and is critical for Sertoli-cell differentiation (Morais da Silva *et al.*, 1996).

The *SRY* gene is among the limited number of genes located on the Y chromosome. If this gene is missing from the Y chromosome, XY cattle develop and express gonadal hypoplasia (Kawakura *et al.*, 1997). Mosaicism in a heifer carrying predominantly XY cells seems to be a cause of non-developed gonads, malformation of Müllerian ducts and infertility (Pinheiro *et al.*, 1990). Detection of the bovine fetal SRY sequence from the peripheral blood of pregnant cows by polymerase chain reaction (PCR) analysis is possible and allows the prediction of sex (Yang *et al.*, 1996).

The testis-specific protein, Y-encoded gene (*TSPY*) is one more bovine gene that has been studied recently. It is expressed in fetal bovine testes at about 6–7 months of age and in some adult tissues, shows a differential splicing and is believed to act during spermatogonial proliferation (Vogel *et al.*, 1997).

Both Y and X chromosomes contain transcriptionally active amelogenin genes, which are highly conserved enamel-matrix proteins, which may not have obvious relations with sex determination. An analysis of amelogenin

mRNA during bovine tooth development was conducted recently (Yuan *et al.*, 1996).

The cycle of the X chromosome

Since the first publication (Lyon, 1961), it has been commonly accepted that one X chromosome in mammalian females undergoes inactivation during early embryonic development. Numerous investigations shed light on different aspects of X chromosome behaviour, including random inactivation in the ICM, preferential inactivation of the paternal X chromosome in the trophoblast and molecular mechanisms of inactivation, to name a few. These data seem fully applicable to the cycle of the X chromosome in cattle, but experimental evidence remains to be seen. The study of the X bivalent in fetal bovine oocytes allows one to hypothesize that changes occur in the transcriptional status that involve activation, inactivation and reactivation of the X chromosomes during embryonic and ovarian differentiation in the conceptus (Koykul *et al.*, 1997).

Defects of sex determination as a result of interactions with other genes

The *roan* locus in Belgian Blue Cattle gives a good example of such an interaction, being involved in determination of the previously mentioned white heifer disease. This disorder is characterized by a group of anomalies of the female genital tract, which stem from abnormal development of the Müllerian ducts. This includes numerous anatomical lesions in the vagina, cervix and uterus and consequent disturbances in their function. Interestingly, no disorders were found in the ovaries. The *roan* locus was mapped to bovine chromosome 5, and the candidate *Steel* gene coding for the mast-cell growth factor, was mapped in the same interval (Charlier *et al.*, 1996).

Another example of interaction between coat-colour locus and sex differentiation is gonadal hypoplasia, found in white Swedish Highland cattle (Lauvergne, 1970). Additional information can also be obtained from Chapter 3.

Freemartinism in Cattle

Phenomenology

A freemartin is a sterile heifer (genetic female, XX) born co-twin to a bull. The frequency of male–female twin pairs is about 50% of the total number of non-identical twins, as expected. With the exception of bovine freemartinism, intersexuality is rarely reported in domestic animals (Cribiu and Chaffaux, 1990). Freemartinism results from the sexual modification of a female twin by

in utero exchange of blood with its male co-twin. The dramatic reduction in the size of the uterus and oviducts in freemartin cattle (Khan and Foley, 1994) is due in part to the female fetus exposure to AMH from the male fetus (Vigier *et al.*, 1984). Between 50 and 80 days, Müllerian duct regression occurs simultaneously in males and freemartins and positively correlates with serum AMH concentrations. However, gonadal production of AMH in freemartins was very low (Vigier *et al.*, 1984). Beyond day 70 of fetal life, gubernacular development in freemartins shows male characteristics (van der Schoot *et al.*, 1995), and the ovaries produce abnormally high amounts of testosterone and little or no oestradiol (Shore and Shemesh, 1981). There are indications that the testes of the male co-twin to a freemartin display abnormal steroidogenesis, being, for instance, responsive to LH stimulation (Shore *et al.*, 1984). However, the majority of these males do not differ from normal bulls later. Interestingly, transgenic female murine fetuses expressing the human *AMH* gene have regressed Müllerian ducts and reduced ovarian aromatase activity (Lyet *et al.*, 1995).

Specific features of the bovine placenta

As mentioned earlier, the placenta in cattle is comprised of specialized areas on the fetal chorion, called cotyledons, which are in direct contact with the uterine epithelium of the dam at specialized areas, called caruncles. Oxygen and nutrients pass from the maternal blood to the blood of the fetus, and waste products pass from the fetal blood into the blood of the dam. However, these connections prevent exchange of fetal and maternal blood. Shortly after the implantation of twin pregnancies in cattle, chorionic fusion and vascular anastomosis of the two fetuses usually occur (Mellor, 1969). This allows for the exchange of blood-forming cells between fetuses, the consequence of which, in heterosexual twins, is the formation of a chimerism (60, XX/XY) in peripheral-blood mononuclear leucocytes (Dunn *et al.*, 1968; Basrur *et al.*, 1970).

XX/XY chimerism

Sex-chromosome chimerism in blood leucocytes of bovine freemartins has been well documented (Eldridge and Blazak, 1977; Mayr and Hager, 1978; Summers *et al.*, 1984; Murakami *et al.*, 1989; Cribiu and Chaffaux, 1990). Interestingly, 100% of Friesian–Brahman-cross twins were chimeric, whereas 50% of Jersey–Brahman-cross twins were chimeric (Summers *et al.*, 1984), indicating that breed type may influence the incidence of freemartin chimerism. Most studies indicate that the sex-chromosome chimerism is found in the blood cells of heterosexual twins but not in the germ cells (Vigier *et al.*, 1973; Ford and Evans, 1977).

Diagnosis of freemartinism and practical aspects

Several methods have been described for the detection of freemartinism (van Haeringen and Hradil, 1993), including sex-chromatin karyotyping (Bhatia and Shanker, 1985; Khan and Foley, 1994; Zhang *et al.*, 1994), blood-group serology (Justi *et al.*, 1995) and using PCR (Schellander *et al.*, 1992; Olsaker *et al.*, 1993; Justi *et al.*, 1995). Of these reported methods, the PCR method is rapid and very sensitive and the most suitable for routine testing (Aasen and Medrano, 1990; Horvat *et al.*, 1993; Justi *et al.*, 1995). The PCR method can also be used to detect the sex of bovine preimplantation embryos (Kirkpatrick and Monson, 1993; Ennis and Gallagher, 1994; Hyttinen *et al.*, 1996).

Some breeding programmes aim to reduce the costs of rearing calves. This encourages selection for twins in cattle and eventually increases the number of sterile freemartin heifers (Kastli and Hall, 1978). Introduction of modern diagnostics has suggested that about 17.5% of heifers born with male co-twins may not be freemartins (Zhang *et al.*, 1994), but further work on a larger number of animals is needed to verify this suggestion. Implementation of the diagnostics may preserve thousands of calves with high breeding value and prevent unnecessary economic losses (Zhang *et al.*, 1994). From a production point of view, slaughter-age freemartins do not differ from normal females in growth traits (Hallford *et al.*, 1976; Gregory *et al.*, 1996), but freemartins have higher marbling scores than normal females (Gregory *et al.*, 1996).

Totipotency and Cloning

The recent reports on the cloning of sheep (Wilmut *et al.*, 1997) and later cattle (Kato *et al.*, 1998) using a nuclei from adult cells shake the ground of the current interpretation of totipotency. It has been well known since classical experiments conducted by R. Briggs and T.J. King that nuclear transplantations can be successful, at least in frogs. Later, Gurdon *et al.* (1975) reported successful transplantation of nuclei from cells derived from adult frogs. However, adult frogs never appeared in these experiments. More recent experiments with frogs have shown that differentiated somatic nuclei transferred to the cytoplasm of oocytes at first meiotic metaphase display enhanced genomic and developmental potential over those transplanted to diplotene oocytes and eggs, at least for the three nuclear cell types tested from the peripheral blood (Di Berardino and Orr, 1992).

The development of nuclear transplantation methods for mammals in the early 1980s resulted in numerous successful experiments (see Chapter 14). The general knowledge obtained from these studies showed that the ability of nuclei to provide normal development decreases significantly from earlier to later stages. This was generally considered as an indication of the progressive loss of totipotency during development and did not contradict data previously obtained with frogs.

Cloning mammals using a nucleus from an adult cell shows that epigenetic changes, which occur in the nucleus during development, can be reversible under certain conditions. Does this mean that the discussion about the loss and restoration of totipotency in consecutive developmental cycles is over? Perhaps not, because in natural conditions nuclei do not jump from one cell to the other, as in transplantation experiments, but stay in differentiated cells. Even if the germ cells are considered, one may assume them to be well-differentiated cells prepared for carrying out a unique function – meiosis. The above notion may be separated into two topics: totipotency of a cell and totipotency of a nucleus artificially transplanted in an egg cell. Meanwhile, significant efforts have been put into the development of totipotent bovine embryonic cell cultures, which may have a great value in cattle breeding (Chapter 14). Thus, regular loss and restoration of totipotency, based on the acquisition and erasing of epigenetic signals, may be a normal feature of the life cycle in many animal species (Ruvinsky, 1997).

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