IN VIVO AND *IN VITRO* ANALYSIS OF TGF-β1 KNOCKOUT EMBRYOS

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SUMMARY

The transforming growth factors-type β (TGF-βs) are a group of proteins expressed in the developing embryo and the adult. Three TGF-β's exist in vertebrates, TGF-β1, TGF-β2, and TGF-β3, all of which have growth inhibitory and stimulatory roles *in vitro,* depending on the target cell type.

In the mouse embryo TGF-β1 RNA expression is first detected in the haemangioblasts of the yolk sac at approximately 7 dpc. These are small cell populations which give rise to the vascular endothelial cells and the haematopoietic cells. Later in development expression continues in tissues undergoing haematopoiesis and vasculogenesis, and is also found during osteogenesis, and in epithelia involved in epithelial / mesenchymal transformations (Akhurst *et al*, 1990). This project was undertaken to investigate the function of TGF-β1 during development by depletion of the protein using molecular methods, such as antisense oligonucleotide technology, in conjunction with post-implantation embryo culture. However, to ensure that any resulting phenotypes were as a result of the removal of TGF-β1, the medium also had to be depleted of TGF-β.

Dulbeccos modified Eagles medium (DMEM) plus rat serum is commonly used for embryo culture, and was also examined in this project. However, analysis of the TGF-β content in the rat serum using the CCL64 assay revealed that considerable amounts of the protein were present (≥15ng/ml), thereby rendering serum unsuitable for use in this project. Therefore, another medium had to be found. Although previous reports had found plasma unsuitable for embryo culture, it was also reported that this biological fluid contained negligible amounts of TGF-β. Analysis of rat plasma using the CCL64 assay in this project detected ≤10pg/ml, suggesting that plasma was ideal for use as a medium depleted of TGF-β1. However, when the rat plasma was combined with DMEM, embryonic development was severely disrupted. Therefore, a completely novel medium was devised, utilising Foetal Mouse Palate medium, in combination with rat plasma. This medium contained no, or undetectable levels of TGF-β, and supported normal development of 8.5 dpc embryo for 24 hours *in vitro.*

Before the culture system could be used with antisense oligodeoxynucleotides to remove TGF-β1 from mouse embryos, two other groups generated transgenic mice with targeted disruption of the TGF-β1 gene (Shull *et al*, 1992; Kulkarni *et al*, 1993). 50% of the TGF-β1 homozygous null mice were born, but only survived to approximately 3 weeks of age before dying of a wasting syndrome involving a

multifocal, mixed inflammatory cell response, and tissue necrosis. No other gross phenotypes were observed. The other 50% of TGF-β1 nulls died *in utero*. In collaboration with Dr.M.Dickson, I determined the reason for this embryonic lethality.

By performing detailed studies at different developmental stages, it was concluded that the embryonic lethality was due to yolk sac abnormalities arising at 9-9.5 dpc. Both the vasculature and the blood was affected. The vasculature defects included weak vessels, a disorganised vasculature, or a complete lack of vessels altogether. Anaemia was observed in a number of yolk sacs. At 9.5 dpc, only if the yolk sac was severely defective was the development of the embryo *per se* affected. Dr.M.Dickson observed that by 10.5 dpc, the embryos appeared necrotic and were close to death (Dickson *et al*, 1995).

Further experiments were performed to examine the dichotomy in embryonic survival. It was suggested that maternal TGF-β1 was rescuing those TGF-β1 null embryos that survived gestation (Letterio *et al*, 1994). This was examined in the current study by culturing embryos of all genotypes in the medium depleted of TGF-β. The intact conceptuses were cultured from 8.5 dpc, when they are all phenotypically normal, to 9.5 dpc, when the defective phenotypes are observable. An increase in the number of abnormal phenotypes was expected if maternal TGF-β was responsible for embryonic rescue. This was not observed. However, it was possible that the maternal TGF-β had already had effect before 8.5 dpc.

The initial effect of TGF-β1 therefore appears to be in yolk sac vasculogenesis and haematopoiesis, coinciding with the expression data. As TGF-β1 was thought to be a potent growth inhibitor of many cell types from *in vitro* studies (Roberts & Sporn, 1990, for review), BrDU incorporation was employed to examine cellular proliferation rates in the TGF-β1 knockout conceptuses. The cells of the yolk sac, and two types of embryonic epithelial cell types were examined. There was no increase in cell proliferation in the TGF-β1 null samples in comparison with the wild-type samples. It is therefore likely that TGF-β1 has a role in the control of yolk sac endothelial and haematopoietic cell differentiation.

TGF-β1 therefore appears to be crucial for normal yolk sac development. Without a functioning yolk sac, the embryo does not receive nutrients and oxygen, and is unable to survive this critical gestation period before development of the chorioallantoic placenta.

DECLARATION

I certify that this thesis does not contain material previously published or written by any other person, except where referred to in the text, and that the results in this thesis have not been submitted for any other degree or diploma.

Julie S.Martin.

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ABBREVIATIONS

Units

aa amino acids	kDa kiloDalton	pg picogram
bp base pairs	L litre	rpm revolutions per minute
cpm counts per minute	M molar	μCi microCurie
cps counts per second	mg milligram	μg microgram
°C degrees Celsius	ml millilitre	μl microlitre
dpc days post coitum	mM millimolar	μm micrometre
g gram	ng nanogram	
kb kilobase	OD optical density	

Chemicals & Reagents

BSA	Bovine serum albumin
BrDU	Bromodeoxyuridine
cDNA	complementary DNA
dCP	doubly centrifuged plasma
DEPC	diethylpyrocarbonate
DMEM	Dulbeccos modified Eagles medium
DNA	Deoxyribonucleic acid
dNTP	deoxynucleotide triphosphates
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
FMP	foetal mouse palate medium
H&E	haematoxylin & eosin
HEPES	N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulphonic acid)
mRNA	messenger RNA
MOPS	3-[N-morpholino]propanesulphonic acid
ODN	oligodeoxynucleotides
PBS	phosphate buffered saline
RNA	ribonucleic acid
rTGF-β	recombinant TGF-β
SDS	sodium dodecyl sulphate
TESPA	3-aminopropyltriethoxysilane

Proteins

BMP	Bone morphogenetic protein
dpp	*decapentaplegic*
DVR	*decapentaplegic*-Vg-related
ECM	extracellular matrix
EGF	epidermal growth factor
FGF	fibroblast growth factor
FN	fibronectin
FSH	follicle stimulating hormone
GDF-9	growth/differentiation factor-9
GDNF	glial-derived neurotrophic factor
LAP	latency associated protein
LTBP	latent TGF-β binding protein
MIS	Müllerian inhibitory substance
TGF-β	transforming growth factor type-beta
Ubx	*Ultrabithorax*
Vg-1	vegetal-1
Vgr-1	Vg-1 related

Other

ASMC	aortic smooth muscle cells
PCR	polymerase chain reaction
UV	ultraviolet
+/+	homozygote wild type
+/-	heterozygote
-/-	homozygote null

Chapter 1

INTRODUCTION.

1.1 Cell-Cell Interactions in Embryogenesis.

Inductive interactions between different cell types were initially identified by Spemann & Mangold in 1924, and it is now known that they occur throughout embryogenesis (Jacobsen & Sater, 1988). Initially, methodological constraints made it difficult to identify the signalling molecules, but with the advent of molecular biology, the methods improved drastically, with the result that a multitude of signalling proteins have since been identified. It has also been possible to compare the molecular components of inductive interactions in different animal models.

Molecular investigations have found that many cell signalling molecules, and other component proteins of their intracellular signalling pathways, appear to be genetically conserved across divergent species, suggesting that they have an important role in biology (Smith *et al*, 1987; Whitman & Melton, 1989, for review). Transforming growth factors type-beta (TGF-β) are one such group of highly conserved, secreted proteins. They belong to a superfamily of more diverse proteins, which have been grouped together due to their high degree of molecular similarity. Although much is already known about the *in vitro* actions and *in vivo* expression patterns of these proteins (Akhurst *et al*, 1990; Roberts & Sporn, 1990), this project examined the function of the TGF-βs, specifically TGF-β1, *in vivo* during murine embryogenesis.

1.2 Transforming Growth Factor Superfamily.

Members of the TGF-β superfamily have been found in a wide variety of species from *Drosophila* to man. The family members have a high degree of molecular homology, with the intron/exon boundaries of every gene essentially identical. Proteins share 40-80% amino acid (aa) homology, and all family members share seven of nine conserved cysteine residues. Between species, the amino acid homology of individual family members can reach 99%. The superfamily has been divided into 5 sub-groups; the TGF-β's, Müllerian inhibiting substance (MIS), *Decapentaplegic*-Vg related group (DVR), the activins, and the inhibins. Additional members which cannot be categorised into any of the above groups have also recently been discovered (Fig.1)

1.2.1 The Transforming Growth Factors Type-beta.

TGF-β1 was molecularly cloned 7 years after initial identification as a co-factor capable of transforming normal rat kidney fibroblasts in culture (Delarco & Todaro, 1978; Derynck *et al*, 1985). Three TGF-βs have now been cloned in vertebrates, TGF-β1, TGF-β2, TGF-β3, and their actions have been extensively studied (Roberts & Sporn, 1990; Akhurst, 1994, for reviews). Each isoform appears to be able to exert inhibitory and mitogenic effects, but the potency varies enormously depending on the isoform. For example, TGF-β1 has a more potent growth inhibitory effect on foetal bovine heart endothelial cells *in vitro* than does TGF-β2 (Muller *et al*, 1987; Jennings *et al*, 1988; Cheifetz *et al*, 1990). TGF-β expression is extensive in both adults and embryos, and will be described in more detail at a later stage.

Molecular analysis of the TGF-βs revealed that all three isoforms are very similar to one another, each containing seven exons, with conserved splice site junctions. They are all synthesised as pre-pro polypeptides, with 390 aa for TGF-β1, and 414 aa for TGF-β2, and 410 aa for TGF-β3 (Derynck *et al*, 1988; Roberts & Sporn, 1990; Pelton *et al*, 1990) (Fig.2). TGF-β2 can undergo differential splicing of the mature domain, to produce pre-pro-peptides of 414 and 442 aa in size (Webb *et al*, 1988). Each isoform contains a 20-23 aa signal peptide at the N-terminus, enabling the protein to be released from the cell. The precursors dimerise, commonly as homodimers (Fig.2). Heterodimers between TGF-β1 and TGF-β2 (Cheifetz *et al*, 1988), and TGF-β2 and TGF-β3 (Ogawa *et al*, 1992) do exist, but are very rare. These pro-peptides undergo proteolytic cleavage at an arginine-rich sequence, 112 aa from the C-terminal, to release the bioactive form of TGF-β. However, the large N-terminal fragment, the

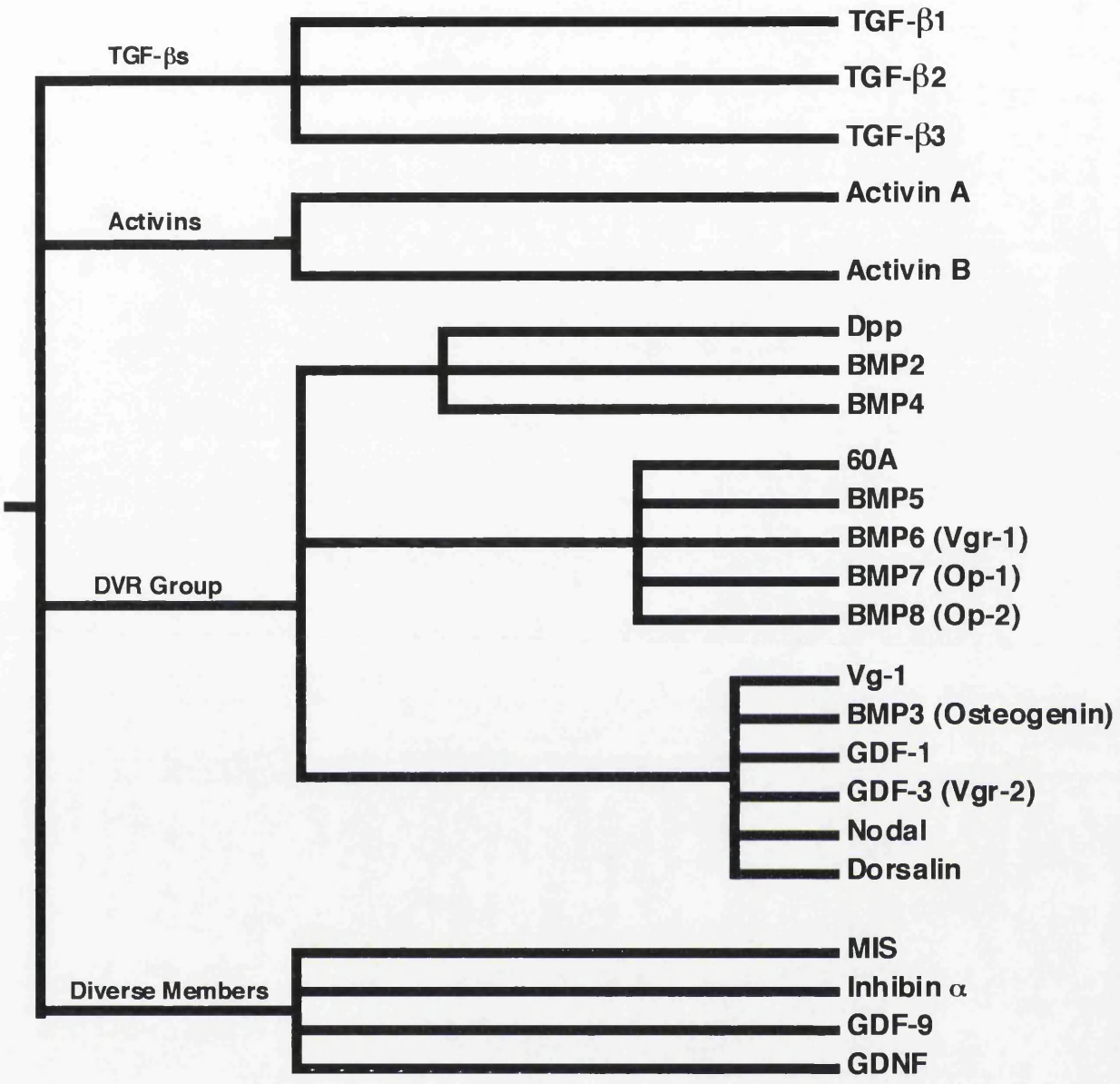


Figure 1 - Transforming Growth Factor Type-Beta Superfamily.

Diagrammatical representation of all known members of the TGF-β superfamily, and the subgroups within. The main branches reveal the four major groups; the TGF-βs, the activins, the DVR group, and a group containing more diverse members which cannot be classified with any of the others.

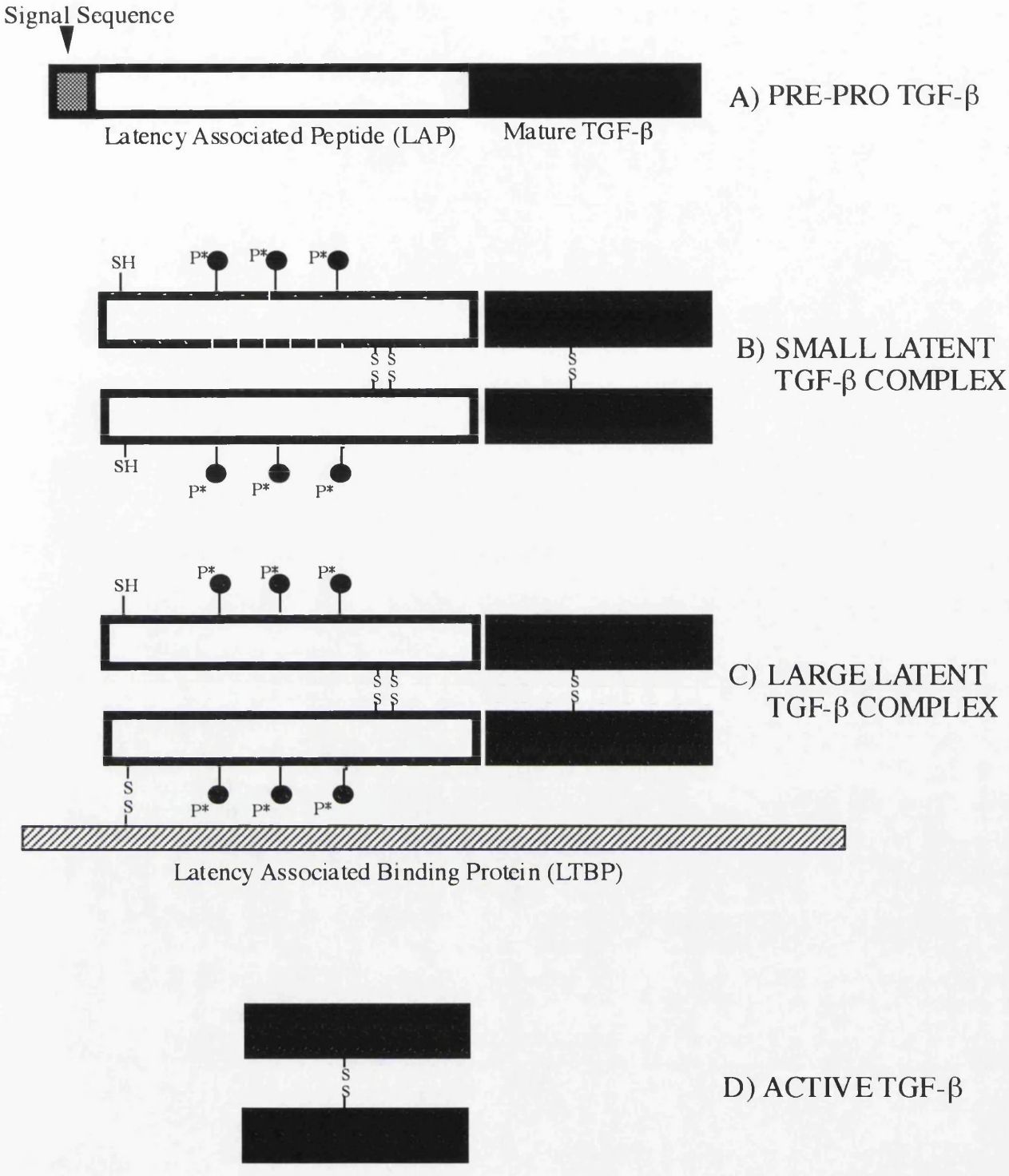


Figure 2 - Diagrammatic Representation of the Different Forms of TGF-β.

TGF-β is initially translated as a pre-pro monomer (A). The NH_2 terminal 20-23 aa encode the signal peptide, followed by approximately 250-310 aa encoding the latency-associated peptide (LAP). The mature region of 112 aa is located at the C-terminal. Two monomers bind to each other by disulphide bonds, and through proteolytic cleavage at an arginine-rich site, the mature regions are cleaved from the LAPs. However, by remaining non-covalently attached, the LAP continues to confer latency. This is the small latent TGF-β complex (B). A further complex exists, termed the large latent complex (C), which has an additional protein, the latency associated binding protein (LTBP). The LTBP cannot confer latency, and only ever binds to the LAP by disulphide bond(s). Mature TGF-β is active once released from the LAP (D). Activation may involve mannose-6-phosphate (P*), plasmin, the LTBP, and transglutaminase.

latency-associated peptide (LAP), remains non-covalently associated with the bioactive dimer, retaining the TGF-β in a latent state, the small latent complex.

A possible significant difference between the isoforms is the existence of a potential integrin binding site in the LAP of TGF-β1 and TGF-β3, which is not present in TGF-β2 (Ruoslahti & Pierschbacher, 1987). However, mature bioactive TGF-β2 also has a specific amino acid sequence that is not present in either TGF-β1 or TGF-β3 (Qian *et al*, 1992). This sequence appears to specify the requirement for betaglycan, a TGF-β type III receptor, which enables TGF-β2 to bind to type I or II receptors (see section 1.3.2). Endothelial and haematopoietic cells both lack betaglycan, and although they respond to TGF-β1 or TGF-β3, they are unable to respond to TGF-β2 (Ohta *et al*, 1987; Ottman & Pelus, 1988; Merwin *et al*, 1991; Qian *et al*, 1992). However, in general, the isoforms are interchangeable in most bioassays (Greycar *et al*, 1989; Roberts *et al*, 1990).

Interspecies homology of the individual TGF-β isoforms is greater than intraspecies homology between the different isoforms. Mature TGF-β1 is identical in human, avian, porcine, simian, and bovine (Derynck *et al*, 1985, 1987; Sharples *et al*, 1987; Van Obberghen-Schilling *et al*, 1987; Jakowlew *et al*, 1988), and there is only one amino acid different between man and mouse (Derynck *et al*, 1986). TGF-β2 and TGF-β3 also show extreme conservation between different species (Seyedin *et al*, 1985; de Martin *et al*, 1987; Cheifetz *et al*, 1987; Madisen *et al*, 1988; Derynck *et al*, 1988, ten Dijke *et al*, 1988). This evidence suggests that the function of each individual TGF-β could be comparable across the species. However, there may be differences in the regulation and utilisation of the proteins in different species during embryogenesis. For example, the involvement of TGF-β3 during the crucial stages of heart development. TGF-β3 is expressed in the chick heart (Runyan *et al*, 1992), whereas TGF-β2 is the predominant isoform expressed in the developing mouse heart (Millan *et al*, 1991; Dickson *et al*, 1993).

1.2.2 Müllerian Inhibiting Substance.

MIS is a distantly related member of the TGF-β superfamily, involved in the development of the reproductive system. During mammalian development, male and female embryos have both a Wolffian and a Müllerian duct. The Wolffian duct regresses in the female but, in the male, under the influence of testosterone, it develops into the vas deferens, seminal vesicles, and epididymus. The Müllerian duct develops into the oviducts and uterus of the female, whereas in the male, MIS, a protein produced in the sertoli cells of the testis, acts to cause Müllerian duct

regression (Josso, 1992; Blanchard & Josso, 1974). The MIS is a disulphide-linked dimer of 140 kDa, the subunits of which are disparate in size (70 and 74 kDa), possibly as a result of different post-translational modification (Cate *et al*, 1986).

Like the other TGF-β superfamily members, MIS is initially produced as a large precursor protein, which can be post-translationally cleaved to form smaller homodimers. However, unlike the other family members, MIS is predominantly active as this large precursor form, and the significance of post-translational cleavage has not yet been established.

There is significant homology in MIS amino acid sequences across different species. For example, bovine and human MIS protein share 78% aa homology, with identity between 108 of the last 112 aa. When MIS is compared with the mature form of the TGF-βs, sequence homology is restricted to the processed C-terminal.

1.2.3 *Decapentaplegic*-Vg Related Group (DVR group).

Members of the DVR group have been classified together as they are more similar to each other than to any of the other superfamily members (Lyons & Moses, 1990). They include the *Drosophila* gene, *Decapentaplegic* (*dpp*), the bone morphogenetic proteins (BMPs), *Xenopus Vg-1*, and its mammalian homologue, *Vgr-1*.

1.2.3.1 *Decepentaplegic.*

In *Drosophila* embryos, *dpp* is involved in the development of the imaginal discs, the wings, the gut, and the dorsal/ventral axis. *dpp* exerts its effect by inducing the expression of certain homeobox genes, thereby specifying the fate of certain cells (Panganiban *et al*, 1990; Reuter *et al*, 1990). For example, *dpp* and the homeobox *Ubx* are thought to positively regulate one another during the development of the midgut (Panganiban *et al*, 1990). If *dpp* or *Ubx* is absent, the midgut develops abnormally. *dpp* is therefore required for normal embryogenesis in *Drosophila*.

Comparison of the amino acid sequence of *dpp* with other members of the TGF-β superfamily shows extensive homology between *dpp* and the bone morphogenetic proteins 2 and 4. These BMP proteins are now thought to be the mammalian homologues of *dpp* (Gelbart, 1989; Wall & Hogan, 1994). The *dpp* amino acid sequence contains the common features of all the members of the superfamily; the conserved C-terminal 112 aa, and the arginine rich region thought to be involved in proteolytic processing of the protein (Gelbart, 1989).

1.2.3.2 Xenopus *Vg-1* and Mammalian *Vgr-1*.

Another member of the DVR group is *Vg-1*. Found in *Xenopus*, *Vg-1* is translated from maternal mRNA localised in the vegetal region of the oocyte (Weeks & Melton, 1987). A closely related gene was recently discovered in mammals by Lyons *et al* (1989) and termed *Vgr-1* for Vg-related. As for the other superfamily members, both *Vg-1* and *Vgr-1* contain the common sequence features of the family.

During *Xenopus* mesoderm formation, a soluble factor released from the endoderm (vegetal pole) induces the overlying equatorial cells to become mesoderm (Nieuwkoop, 1969). The temporal and spatial expression of *Vg-1* suggests that it is a candidate for this inducing signal (Weeks & Melton, 1987). When explanted animal poles are treated with mature *Vg-1* protein dorsal mesoderm is induced, with expression of the corresponding markers (Kessler & Melton, 1995). *In vitro* assays have also shown the activins, bone morphogenetic protein 4 (BMP 4), and several fibroblast growth factors (FGFs) to be capable of inducing mesoderm formation. Furthermore dominant negative mutation of the activin receptors type I and II, BMP receptor type II, or FGF receptor 1 block this inductive activity (Amaya *et al*, 1991; Hemmati-Brivanlou & Melton, 1992; Graff *et al*, 1994; Nakamura *et al*, 1992; Suzuki *et al*, 1994). This probably occurs by interaction of the dominant negative with an endogenous *Vg-1* specific receptor (Kessler & Melton, 1995).

Another postulated role for *Vg-1* is in the formation of the Nieuwkoop centre (Thomsen & Melton, 1993) The Nieuwkoop centre is an area of dorsal vegetal cells in the 32-cell blastocyst, which, when transplanted into the ventral vegetal region, can induce the formation of an entirely new body axis (Gimlich & Gerhart, 1984; Kageura, 1990). This occurs by the induction of dorsal mesoderm by the Nieuwkoop centre, which then forms, amongst other structures, Spemann's organiser, resulting in axial formation (Elinson and Kao, 1989; Gerhart, *et al*, 1989). The hypothesis that *Vg-1* is involved in the formation of this centre arises because the initial cortical rotation during the first cell cycle of the *Xenopus* embryo would relocalize *Vg-1* from the vegetal cortex to the region of the future Nieuwkoop centre. Also, an injection of mature *Vg-1* can rescue UV ventralised embryos, inducing the formation of the Nieuwkoop centre, and returning the axes to normal (Thomsen & Melton, 1993). However, further experiments are being performed to determine if *Vg-1* is endogenously involved in this process.

1.2.3.3 Bone Morphogenetic Proteins.

BMP's were first identified by Urist (1965), although at that time the proteins were not purified. Urist implanted extracts of demineralised bone into ectopic sites in rodents, and found that new bone formed. Mesenchymal cells invaded the implant and differentiated into chondrocytes, followed by the differentiation of osteoblasts and osteoclasts. The inducing component in the extract was identified as protein, but whether it was a single protein or many working together was not known.

In 1988 Wozney cloned the BMP cDNAs and identified these proteins as the inducers of bone formation. Eight BMP's have since been isolated, seven of which are related to the TGF-β superfamily of proteins, and belong in the DVR group (Wozney *et al*, 1988). Mature BMP 2 and 4 are 75% identical to *Drosophila dpp* protein, and therefore are classified along with *dpp*, while BMP 3, 5, 6, 7, and 8 are in other subgroups of DVR (Fig.1).

The RNA expression pattern of BMP 2 during embryogenesis was found to overlap with patterns for other TGF-β superfamily members (Lyons *et al*, 1990). However, this is not conclusive evidence for interaction between BMP 2 and the other members, and may actually represent functional redundancy.

1.2.4 The Activins.

Activins are dimers of two β chains, and exist as activin A ($\beta_a\beta_a$), activin B ($\beta_b\beta_b$), and activin AB ($\beta_a\beta_b$). As well as promoting the release of follicle stimulating hormone (FSH) from the pituitary (Mason *et al*, 1985), studies have shown that the activins are capable of inducing mesoderm formation, and structures derived from mesoderm (Albano *et al*, 1990; Smith *et al*, 1990; Mitrani *et al*, 1990; Mitrani & Shimoni, 1990; Nakamura *et al* , 1992). Expression studies have also shown that the activins are expressed in several tissue types during postimplantation development (Feijen *et al*, 1994). At 6-9.5 dpc the subunits are expressed exclusively in the decidua. However, by 10.5 dpc activin A is found in the mesenchymal tissues of the embryo, in the same tissues, or adjacent to tissues expressing TGF-βs. Activin B is expressed in the central nervous system at this stage. There are several regions where both the β_a and β_b subunits are expressed, including the oesophagus, the mesenchyme of blood vessel walls, and the pleuroperitoneal membrane, which could suggest that all three activin isoforms are present. By comparing this data with activin receptor expression patterns, Feijen *et al* (1994) suggested that activin A could be involved in craniofacial development, and the formation of the inner ear, the tongue,

and the lung; activin B in the development of the central nervous system, the stomach, and the eyelids; and activin AB in the development of the limb and shoulder.

Knockout mice deficient in activin A, B, AB, or the activin type II receptor were recently generated, and all mutants developed to term (Vassalli *et al*, 1994; Matzuk *et al*, 1995a,b). The initial observation was that zygotic activins were not required for mesoderm formation (Albano *et al*, 1990; Smith *et al*, 1990; Nakamura *et al*, 1992). However, as occurs with TGF-β1 knockout mice, it is possible that there is maternal protein crossing the placenta which rescues the mutant embryos at this stage (Letterio *et al*, 1994). Mice which lacked activin A, or A and B, exhibited the same phenotype; they lacked whiskers, lower incisors, and had defects in their secondary palate resulting in failure to suckle and difficulty in breathing. The mice died at approximately 24 hours *post-partum* (Matzuk *et al*, 1995a). The defects are consistent with the observed expression of activin A in development (Feijen *et al*, 1994), and *in vitro* effects of the activins on chondrogenesis and osteogenesis (Centrella *et al*, 1994; Luyten *et al*, 1994). Activin B mutants were viable but had reduced female fertility and defective eyelid development (Vassalli *et al*, 1994).

The activin type II receptor knockouts were expected to exhibit a phenotype like the activin mutants, but although there were a few with facial and skeletal abnormalities, the vast majority were overtly normal, with only a decrease in the levels of FSH, and reduced reproductive capabilities (Matzuk *et al*, 1995b). This data suggests that the activins signal through the activin type II receptor for their FSH activity, but not for their role in embryonic development. As deletion of activin B also resulted in reduced fertility this may be the only isoform to signal through the activin type II receptor, and other receptor heterodimers may be required for activin A and AB. However, this data is difficult to interpret for activins alone because mutations in either β subunit also disrupts the expression of the inhibins.

1.2.5 The Inhibins.

Inhibin exists as two heterodimeric isoforms, inhibin A ($\alpha\beta_a$) or inhibin B ($\alpha\beta_b$), consisting of one inhibin α subunit, plus one of the two activin β subunits (β_a or β_b). The α chain is relatively distinct, whereas the β chains exhibit 46% and 38% homology to TGF-β.

Inhibin functions as a hormone to inhibit the release of FSH from the pituitary, thereby preventing the expulsion of an oocyte from the ovary (Mason *et al*, 1985).

The inhibin subunit has also been identified in other tissues such as brain, kidney, placenta, adrenal gland and the pituitary, and it is therefore likely that inhibins are growth and/or differentiation factors as well as hormones (Meunier *et al*, 1988). However, mice deficient for inhibin α were viable, and only developed gonadal stromal tumours (Matzuk *et al*, 1992). This observation suggests that either the widespread expression of inhibin α represents non-functional protein and the only function of inhibin is as a reproductive hormone, or that there is functional redundancy with other molecules in the knockout mice. The mild phenotype of the α mutants also suggests that the defects observed in the activin mutants were as a result of the removal of the activin isoforms, and not the inhibin isoforms (Matzuk *et al*, 1995a,b).

1.2.6 Recent Additions to the Superfamily.

There are several new additions to the TGF-β superfamily, most belonging to the DVR subgroup (Fig.2). They include 60A, a *Drosophila* BMP (Doctor *et al*, 1992); *dorsalin-1*, and *nodal* (Basler *et al*, 1993; Zhou *et al*, 1993), both of which are thought to be involved in the dorsal/ventral patterning of the mouse embryo; and growth/differentiation factor-3/Vg-related 2 (GDF-3/Vgr-2) (Jones *et al*, 1992). GDF-9, and glial-derived neurotrophic factor (GDNF) are also recent additions, but cannot be classified into any of the existing subgroups (McPherron & Lee, 1993; Lin *et al*, 1994). Further research is required to identify the exact roles of these proteins, especially during development where they are found to be expressed in embryonic tissues undergoing inductive interactions and patterning.

Based on sequence similarities, new members of the TGF-β superfamily continue to be identified, but their functions are as yet unknown. The TGF-βs however, have obviously been studied over a longer period of time, and a plethora of molecular and functional information has already been obtained for these proteins.

1.3 Transforming Growth Factors Type-beta Latency, Activation, and Ligand/Receptor Interaction.

1.3.1 TGF-β Latency.

As discussed in section 1.2.1, TGF-β is synthesised as a large precursor, which is proteolytically processed to form an inactive complex, held together by hydrophobic bonds (Fig.2). This complex, which contains the TGF-β gene product alone, is termed the small latent complex.

Another form of latent TGF-β is released from platelets (Assoian & Sporn, 1986; Fava *et al*, 1990) (Fig.2). This form is termed the large latent TGF-β complex and contains a third component called the latent TGF-β binding protein (LTBP) (Miyazono *et al*, 1988, Wakefield *et al*, 1988, Okada *et al*, 1989). LTBP is a separate gene product of 125-160 kDa, and is bound to one of the LAP monomers by disulphide bonds. LTBP is only ever bound to latent TGF-β via the LAP, never to mature TGF-β. Furthermore, LTBP cannot confer latency alone. This has been shown by incubation of ^{125}I-labelled active TGF-β with LTBP followed by the immunoprecipitation of the TGF-β with antibody. LTBP was not observed to have bound to any of the mature TGF-β (Kanzaki *et al*, 1990). The LAP is sufficient to confer latency (Miyazono *et al*, 1993).

LTBP cDNA was cloned from human foreskin fibroblasts and rat kidney cDNA libraries (Kanzaki *et al*, 1990; Tsuji *et al*, 1990). There are 1394 aa in the open reading frame of the human LTBP. When the cDNA was transfected into COS cells or HEL cells, the resulting protein was 190-205 kDa (Kanzaki *et al*, 1990; Miyazono *et al*, 1991). This suggests that the 125-160 kDa LTBP retrieved from platelets must have sustained proteolytic processing.

The amino acid sequence of the LTBP is highly repetitive, and consists of sixteen epidermal growth factor (EGF)-like repeats and three "LTBP-like" repeats (Kanzaki *et al*, 1990; Tsuji *et al*, 1990). These cysteine-rich repeats constitute more than 60% of the LTBP protein. EGF-like repeats have been observed in a wide variety of proteins that are functionally unrelated, such as blood coagulation factors, growth factors, homeotic gene products, receptors, and extracellular matrix proteins (Davis, 1990). The presence of consensus amino acid sequences in some of the EGF-like repeats suggests that Ca^{2+} can bind to the LTBP (Colosetti *et al*, 1993). The function of the EGF-like regions is not fully understood, but it has been postulated that they

may be involved in homophilic and/or heterophilic protein interactions. *In vitro*, cells which express *Notch*, a *Drosophila* protein with EGF-like repeats, are found to aggregate with other *Notch*-expressing cells or even other proteins that contain EGF-like repeats. This occurs in a Ca^{2+}-dependent manner (Rebay *et al*, 1991).

The other repeats in the LTBP are the "LTBP-like" motifs, which consist of eight cysteine residues. They are observed three times in LTBP, and have also recently been found in a related protein, fibrillin (Maslen *et al*, 1991). Fibrillin is a 350 kDa glycoprotein and is a constituent of the extracellular matrix (ECM) in elastic tissues. A genetic defect associated with abnormalities in fibrillin is Marfan's syndrome. This syndrome is typified by skeletal abnormalities, dislocation of the lens of the eye, and aortic aneurysm and dissection. Immunohistochemical studies had shown that LTBP was frequently localised in connective tissues (Waltenberger *et al*, 1993; Mizoi *et al*, 1993), and so Taipale *et al* (1994) investigated whether LTBP was also a constituent of the ECM, or just interacted with matrix components. The evidence demonstrated that the LTBP binds to the ECM and enables TGF-β to associate with the ECM after secretion without any activation step. Release of the TGF-β is via proteolytic cleavage of the LTBP (Taipale *et al*, 1994).

1.3.2 TGF-β Activation.

Activation of TGF-β requires the complete dissociation of the LAP from the mature region of the protein. The process by which this occurs *in vivo* is not yet fully understood, although there are several suggestions. Recent studies have proposed a role for mannose-6-phosphate (Dennis & Rifkin, 1991). Mannose-6-phosphate is a signal, targeting proteins for transport to the lysosomes of the cell, and LAP contains mannose-6-phosphate residues (Fig.2) (Purchio *et al*, 1988; Kovacina *et al*, 1989). It is possible that the LAP binds to the mannose-6-phosphate/IGFII receptor, and is redirected either to the cell surface or into a lysosomal compartment for activation (Taipale *et al*, 1994).

It is also feasible that the cation independent mannose-6-phosphate receptor acts indirectly in the activation of TGF-β by limiting the supply of latent TGF-β. This receptor is known to participate in the degradation of the ECM (Brauker *et al*, 1986; Chao *et al*, 1990). If this activity was inactivated by the binding of ligand, ie LAP, then ECM degradation would be prevented, and the supply of matrix-derived latent TGF-β would be restricted. The requirement for the proper assembly of the extracellular matrix is shown by the repression of TGF-β activation when the matrix cross-linking enzyme, transglutaminase, is inhibited (Kojima *et al*, 1993).

Nevertheless, mannose-6-phosphate does appear to be a prerequisite for activation from the finding that latent TGF-β requires binding to the mannose-6-phosphate/IGF-II receptor in co-culture of endothelial cells and smooth muscle cells for activation to occur (Dennis & Rifkin, 1991).

However, the above data only suggests mechanisms for directing the latent form for activation, and not activation itself. Early investigations found that recombinant latent TGF-β1 or TGF-β1 from fibroblast conditioned medium can be activated by plasmin (Lyons *et al*, 1988, 1990), suggesting a role for proteases in the activation process. Plasmin is located at the cell surface, thus the importance of targeting of latent TGF-β to the cell surface by the mannose-6-phosphate receptor, or LTBP becomes more obvious (Flaumenhaft *et al*, 1993). Further evidence for a role for plasmin is the inhibition of TGF-β activation if either plasminogen, or the receptor-bound urokinase are omitted (Odekon *et al*, 1994). Acid conditions (pH3.6), and heat treatment can also activate latent TGF-β in cultured cells (Lawrence *et al*, 1985; Brown *et al*, 1990). However, it is unlikely that these factors are involved *in vivo* .

1.3.3 TGF-β Receptors.

Molecular cloning of the receptors for various members of the TGF-β superfamily has only recently been performed. Three receptor types were originally identified on the basis of their differential sizes in chemical crosslinking studies using radio-iodinated TGF-β. These were Types I (53-65 kDa), II (85-95 kDa), and III (200-400 kDa) (Massagué, 1992; Lin & Lodish, 1993; for reviews).

The first receptor of the TGF-β superfamily to be cloned was the activin type II receptor, ActRII, followed shortly by a second activin receptor, ActRIIB (Mathews & Vale, 1991; Attisano *et al*, 1992; Mathews *et al*, 1992). The sequencing of these receptors identified the first transmembrane serine/threonine kinase molecules and thus a completely novel form of growth factor receptor. All of these proteins contain an extracellular domain, a transmembrane region, and an intracellular domain. This latter region contains the serine/threonine kinase domain.

The TGF-β type II receptor was the next serine/threonine kinase receptor to be identified (Lin *et al*, 1992), and was found to have limited homology to the activin receptor in the extracellular domain (<10%), but extensive homology in the intracellular domain (47%). This evidence suggested that the TGF-β superfamily signalled through a novel type of receptor group, the serine/threonine kinases, and not the more common tyrosine kinases.

On identification of the type I receptors, their intracellular regions were also found to contain serine/threonine kinase domains (Attisano *et al*, 1993; Ebner *et al*, 1993; Franzen *et al*, 1993). However, these kinase sequences were different than those of the type II receptors, and therefore the receptors were classified into a separate subgroup.

Type I receptors so far identified include ActRI, ActRIB, BMPRI, BMPRIB, TβRI, and TSR (Frolik *et al*, 1984; Attisano *et al*, 1993; ten Dijke *et al*, 1993; Carcamo *et al*, 1994). Type II receptors include ActRII, ActRIIB, TβRII, and BMPRII (Mathews & Vale, 1991; Lin *et al*, 1992; Attisano *et al*, 1992; Estevez *et al*, 1993).
Although both type I and type II receptors contain potential signalling sequences, recent studies have shown that both receptor types are required for signalling to occur. An exception to this rule are the type I and type II BMP receptors, *daf-1* and *daf-4* (Georgi *et al*, 1990; Estevez *et al*, 1993). It appears as if *daf-4* is capable of binding the ligand and transduction of the signal without *daf-1* (Estevez *et al*, 1993). However, for all of the other receptors identified so far, if either receptor type is present alone, there is no response to ligand binding.

Mutant mink lung epithelial cells with defective TGF-β type I or type II receptors were generated by chemical mutagenesis (Laiho *et al*, 1990, 1991). The R clones had a severe reduction or complete loss of type I receptors, and the DR clones had reductions in both receptors. Wild-type mink lung epithelial cells are inhibited by TGF-β1, but the TGF-β response was not observed when the ligand was applied to either of the mutant clones (Laiho *et al*, 1990, 1991). In the R mutants, the ligand could bind to the type II receptor but there was no signal transduction, whereas in the DR mutants, the TGF-β could not even bind to either receptor. However, if wild-type type I receptors were transfected into the R mutants, the TGF-β response was observed. This was also seen with the transfection of wild-type type II receptors into the DR mutants (Wrana *et al*, 1992; Inagaki *et al*, 1993). These observations suggested that the type II receptor was imperative for signalling, but that it needed the type I receptor in order to do so, and that the type I receptor required the presence of type II to bind the ligand.

As identified by ligand-binding, the study with the DR mutants also suggested that the type II receptor is required for the appearance of the type I receptors on the cell surface (Kingsley, 1994). Antibodies to either receptor type will coimmunoprecipitate both type I and type II receptors after crosslinking in the presence of ligand (Wrana *et al*, 1992; Attisano *et al*, 1993; Franzen *et al*, 1993) suggesting that they form a

heteromeric complex, but whether this exists only in the presence of ligand is not yet known.

As well as TGF-β type I and type II receptors, there are type III receptors. Betaglycan was first to be identified (Lopez-Casillas *et al*, 1991; Wang *et al*, 1991), and will be discussed below. Endoglin is also a type III receptor (Cheifetz *et al*, 1992), and much of the information regarding betaglycan also refers to endoglin. However, endoglin differs from betaglycan in that it is only expressed in vascular endothelial cells, it can only bind TGF-β1 and TGF-β3, and it does not exist in a free floating form.

The type III receptor is often the most abundant TGF-β receptor present on the cell surface with as many as 200,000 molecules per cell (Massague, 1985; Fanger *et al*, 1986; Cheifetz *et al*, 1992). Betaglycan is a membrane protein (Cheifetz *et al*, 1988a,b), but unlike types I and II, type III does not have a serine/threonine kinase intracellular domain. This protein consists of an extracellular domain and the transmembrane region to anchor it into the membrane (Gougos & Letarte, 1990; Lopez-Casillas *et al*, 1991; Wang *et al*, 1991). There is no substantial intracellular region. It consists of only 43 amino acids, 42% of which are serine/threonine residues. The extracellular domain is heavily modified by glycosaminoglycans, but after treatment with chondroitinase ABC and heparitinase, a 120 kDa protein is obtained, the core protein, to which TGF-β binds (Segarini & Seyedin, 1988; Cheifetz *et al*, 1988).

The type III receptor is found in many different cell types, but certain cell types such as haematopoietic progeniter cells (Ohta *et al*, 1987), and endothelial and epithelial cells in primary or early passage cultures (Segarini *et al*, 1989) do not express betaglycan, although they do have the type I and type II receptors. These cells still exhibit a TGF-β response, suggesting that type III receptors are not directly involved in the signalling of TGF-β (Cheifetz *et al*, 1990; Massague *et al*, 1990). Betaglycan binds TGF-β and then presents it to the type II receptor (Lopez-Casillas *et al*, 1993; Yamashita *et al*, 1994).

However, as well as being bound to the membrane, betaglycan can be "shed" and float free (Andres *et al*, 1989; Lopez-Casillas *et al*, 1991). This form can still bind ligand, but does not present it to the signalling receptors (Lopez-Casillas *et al*, 1994). It therefore appears as if betaglycan in this soluble, free-floating form is removing excess ligand, or is a method for controlling the actions of the ligand. It is a dual modulator of TGF-β access to the signalling receptors.

The affinity for ligand varies between the different receptors. Some TGF-β type I and type II receptors bind all three TGF-β isoforms equally, but there are subsets which have a higher affinity for TGF-β1 and TGF-β3 (Cheifetz *et al*, 1990; Segarini *et al*, 1989). It is likely that this subset of receptors is present in haematopoietic and endothelial cells which do not respond to TGF-β2 (Ohta *et al*, 1987; Ottman & Pelus, 1988; Merwin *et al*, 1991; Qian *et al*, 1992). However, in the presence of betaglycan the affinity for TGF-β2 in this subset of type I and type II receptors increases. Betaglycan itself binds to all TGF-β isoforms equally.

It has recently been suggested that type I receptors can interact with different ligands, depending on the type II receptors present, for example, an activin type I receptor will bind activin or TGF-β depending on whether there are activin or TGF-β type II receptors expressed (Attisano *et al*, 1993; Ebner *et al*, 1993). Converse to this, the intracellular response resulting from type II receptor binding is determined by the type I receptor that it complexes with (Attisano *et al*, 1993). This phenomenon is already known to occur with the TSR type I receptor, which binds both activin and TGF-β ligands, and activin and TGF-β type II receptors (Attisano *et al*, 1993).

The evidence so far suggests that a ligand will evoke different cellular responses by binding to different combinations of receptor subunits. The members of the TGF-β superfamily also form homo- or heterodimeric complexes, and it may be that different combinations of ligand subunits drive the formation of specific receptor complexes, which in turn give rise to a specific cellular response (Kingsley, 1994). It is also possible that the cells produce different receptors for the same ligands, thereby giving rise to a diverse range of responses from the same ligand (Kingsley, 1994). Many members of the TGF-β superfamily do exhibit a wide range of actions, and perhaps this is the method through which they do so. Even the actions of the TGF-β isoforms themselves, which have been studied in detail, show great diversity from cell-type to cell-type, and between adults and embryos.

1.4 Actions of Transforming Growth Factors Type-Beta.

1.4.1 The Extracellular Matrix.

Initial expression studies for TGF-β found that the protein was localised in the mesenchyme of embryonic and adult tissues (Ellingsworth *et al*, 1986; Heine *et al*, 1987; Flanders *et al*, 1989; Thompson *et al*, 1989). It is now known that TGF-β

controls growth, differentiation and function of the mesenchymal cells predominantly indirectly *via* the extracellular matrix (Roberts & Sporn, 1990).

Many of the matrix proteins which constitute the ECM are produced in response to TGF-β. Collagen types I, III, IV, and V, chondroitin/dermatan sulphate proteoglycans, fibronectin, osteopontin, thrombospondin, tenascin, and elastin are all upregulated by TGF-β (Seyedin *et al*, 1985; Ignotz & Massague, 1986; Chen *et al*, 1987; Falanga *et al*, 1987; Varga *et al*, 1987; Rossi *et al*, 1988; Madri *et al*, 1988; Hiraki *et al*, 1988; Noda *et al*, 1988; Penttinen *et al*, 1988; Pearson *et al*, 1988; Lui & Davidson, 1988). Normally, to control the amount of ECM present, ECM proteins are degraded by proteases. However, TGF-β also controls this degradation by downregulating the synthesis of proteases, such as plasminogen activator, collagenase, and elastase, as well as inducing the production of protease inhibitors including plasminogen activator inhibitor, and tissue inhibitor of metalloproteinases (Laiho *et al*, 1986; Lund *et al*, 1987; Edwards *et al*, 1987; Overall *et al*, 1989).

One TGF-β effect that has a direct consequence on the cell is the ability of TGF-β1 and TGF-β2 to increase the expression of integrins, cell membrane receptors for extracellular matrix proteins, thereby increasing the interaction of cells with the ECM (Hynes, 1987; Ignotz & Massague, 1987; C.J.Roberts *et al*, 1988). The regulation of fibronectin and its integrin, VLA-5, by TGF-β is a well documented example (McDonald *et al*, 1987; C.J.Roberts *et al*, 1988). By affecting the ECM in these ways TGF-β indirectly and directly affects the behaviour and phenotype of the cells.

1.4.2 Skeletal and Smooth Muscle.

The majority of work investigating the effect of TGF-β on muscle has been performed *in vitro*. The data presented here will focus on skeletal and smooth muscle, while cardiac muscle will be discussed in section 1.4.5.

Initial investigations had suggested that TGF-β was a growth and differentiation inhibitor of skeletal muscle cells (Florini *et al*, 1986; Massague *et al*, 1986; Olson *et al*, 1986). However, this interpretation has since been questioned. When TGF-β1 was added to L6E9 rat skeletal myoblasts in a mitogen-rich medium, the cells were growth inhibited, but were also induced to differentiate into multinucleated myotubes (Zentella & Massague, 1992). This result suggested that under mitogen-rich conditions *in vivo*, TGF-β1 may be an endogenous inducer of myogenic differentiation. However, the effect of TGF-β on smooth muscle cells does not appear to affect differentiation, but proliferation. In porcine aortic smooth muscle cells

(ASMC) the activity of TGF-β is concentration dependent (Hwang *et al*, 1992). At 25pg/ml the cells are growth stimulated, whereas at 100pg/ml or above, they are growth inhibited. In human adult ASMCs, the population doubling time is controlled by endogenously produced TGF-β, acting in an autocrine growth inhibitory loop (Kirschenlohr *et al*, 1993). The doubling time in the human cells is approximately twice that found for adult rat ASMCs, which do not produce TGF-β. Therefore, the action of TGF-β on muscle cells can vary depending on the type of cell, the concentration of TGF-β, and the species from which the cells were obtained.

TGF-βs are also expressed in the developing muscle of the embryo (Pelton *et al*, 1991; Millan *et al*, 1991). TGF-β2 was initially identified in the developing somites at 8.5 dpc by *in situ* hybridisation, and at later stages in the smooth muscle of the body wall (Dickson *et al*, 1993). *In vitro* studies also found that TGF-β can exert an effect on developing limb bud myoblasts. When TGF-β was added to cultured limb buds, embryonic myoblasts formed, but foetal myoblast formation was inhibited. However, if anti-TGF-β antibody was added to the cultured limb buds, foetal myoblasts appeared (Cusella-De Angelis *et al*, 1994). This suggested that the differentiation of foetal myoblasts, as opposed to embryonic myoblasts, is normally controlled by endogenously produced TGF-β. In addition, a role for TGF-β in myogenesis was further substantiated by the finding that TGF-β1 and TGF-β2 are capable of inducing embryonic stem cells to differentiate into cardiac and skeletal muscle cells (Slager *et al*, 1992). Therefore, taken together, the expression patterns of TGF-β2, the response of the ES cells, and the differential response of different myoblasts implies that TGF-β may regulate muscle histogenesis in the embryo.

1.4.3 Bone.

Bone is the most abundant source of TGF-β in the body, containing approximately 0.3mg/kg (Seyedin *et al*, 1986). It is hypothesised that TGF-β has a role in the formation and remodelling of mineralised tissues by acting on mesenchymal precursor cells, chondrocytes, osteoblasts, and osteoclasts (Centrella *et al*, 1988), cells which, in the developing embryo, do express TGF-β1 (Heine *et al*, 1987). TGF-β may also be involved in bone growth, remodelling and repair in the adult, probably by the same methods employed during embryogenesis (Seyedin *et al*, 1985, 1986; Carrington *et al*, 1988). Evidence for the osteoinductive role of TGF-β was obtained when TGF-β was repeatedly injected into the periostea of rat parietal bones, or subperiosteally into the rat femur, resulting in new bone formation (Noda & Camilliere, 1989; Joyce *et al*, 1990). TGF-β is also capable of closing non-healing skull defects in experimental rabbits (Beck *et al*, 1991). One hypothesised action for

TGF-β in bone involves the activation of latent TGF-β in the acidic, proteolytic environment created by osteoclast activity (Oreffo *et al*, 1989). Active TGF-β then inhibits the osteoclasts, but promotes osteoblast activity. Matrix deposition would also occur which would serve as a centre for mineralisation.

1.4.4 Immune System.

In the immune system, TGF-β appears to have an inhibitory effect on numerous cell types. *In vitro,* it inhibits the proliferation of certain cells, such as thymocytes, and T and B lymphocytes (Ristow, 1986; Kehrl *et al*, 1986,a,b,1989); it inhibits the activity of natural killer cells (Rook *et al*, 1986); and inhibits the production of lymphokine-activated killer cells and allospecific cytotoxic T lymphocytes (Mule *et al*, 1988; Espevik *et al*, 1988). TGF-β also has an antagonistic effect on the interleukins IL-1, IL-2, and IL-3, tumour necrosis factor and the interferons (Kehrl *et al*, 1986; Wahl *et al*, 1988; Roberts & Sporn, 1990). A bifunctional action is observed for the effect of TGF-β on IgG secretion; it upregulates IgG secretion from splenic lymphocytes from 0.1% to more than 10%, whereas if B cells expressing IgG are treated with TGF-β, then IgG secretion is inhibited (Coffman *et al*, 1989). Therefore, with the ability to exert this range of inhibitory effects, TGF-β appears capable of extensive immunosuppressive activity.

Further data suggests that, *in vivo,* TGF-β acts as an autoregulatory lymphokine that regulates T-cell clonal expansion, and can also suppress immune cell function (Kehrl *et al*, 1986). Sufferers of glioblastoma often have reduced cell-mediated immune responses (Wrann *et al*, 1987), and on purification of this immune suppressor from a glioblastoma cell line, it was identified as TGF-β2 (De Martin *et al*, 1987). Other TGF-βs may be secreted from other tumours, with the same immune repressing capacity.

Positive activities of TGF-β in the immune system include the chemoattractant effect on monocytes, and at higher concentrations, the stimulation of monocytes to increase the mRNA expression of certain growth factors (Wahl *et al*, 1987; McCartney-Francis *et al*, 1989). TGF-β also inhibits hydrogen peroxide release from macrophages (Tsunawaki *et al*, 1988), which may have a beneficial effect during wound healing by removing the killing capacity of the cells, while allowing the growth factors released from the cells to act.

Potent evidence for the involvement of TGF-β in the immune system was recently obtained when mice deficient for TGF-β1 (TGF-β1$^{-/-}$) were created by homologous

recombination (Fig.3). TGF-β1$^{-/-}$ mice which survived gestation, died approximately 3 weeks after birth (Shull *et al*, 1992; Kulkarni *et al*, 1993). Morphological examination of these mice revealed that there had been massive infiltration of lymphocytes and macrophages into multiple organs, which had led to a wasting syndrome, tissue necrosis, and organ failure, followed by death. It appeared as if the immune system had gone awry.

All of the evidence provided from *in vitro* and *in vivo* studies suggests that TGF-β controls the detrimental aspects of the inflammatory response while helping the anabolic effects of growth factors on tissue repair.

1.4.5 Heart.

During development of the mouse heart, angiogenic clusters and promyogenic precursors located in the mesenchyme on the lateral sides of the presomite embryo, rapidly spread in the cephalic direction, fuse, and form the primitive heart tube at approximately 7.5-7.75 days *post-coitum* (dpc). This tube consists of endocardium and myocardium separated by acellular cardiac jelly. From 7.5 to 11 dpc major morphogenetic movements occur which change this simple heart tube into a convoluted, 4 chambered, contracting organ (Viragh & Challice, 1980).

TGF-β1 and TGF-β2 are the major isoforms expressed in the developing mouse heart at these early stages (Akhurst *et al*, 1990). TGF-β3 is restricted temporally and spatially to 14.5-16.5 dpc in the mesenchymal condensations at the base of the heart valves (Millan *et al*, 1991). TGF-βs 1 and 2 however, have been detected by *in situ* hybridisation as early as 7 dpc in the cardiac mesoderm, and by 7.5-8 dpc TGF-β1 expression was detected in all endocardial components, with TGF-β2 in the myocardium (Akhurst *et al*, 1990; Dickson *et al*, 1993). By 8.5 to 9.5 dpc, although still expressed in the same cell types, TGF-β1 and TGF-β2 expression becomes restricted to the outflow tract and atrioventricular regions. It is at this time that the cardiac jelly in these regions is invaded and replaced by mesenchyme, forming the septae of the heart (Dickson *et al*, 1993).

The precise actions induced by the TGF-βs in heart development are not known, although it has been suggested that during early stages TGF-β2 induces the differentiation of myocardium from cardiomyocytes (Dickson *et al*, 1993). *In vitro* studies do support a differentiative function for TGF-β in cardiac muscle cells. As mentioned previously (section 1.4.2), addition of TGF-β1 or TGF-β2 to ES cells in culture induces the formation of cardiac and skeletal muscle, with TGF-β2 increasing

the beating rate of the cardiac muscle formed (Slager *et al*, 1992). In addition, TGF-β1 induces the expression of genes for myocardial contractile proteins in cultured neonatal rat cardiomyocytes, and augments cardiac cell differentiation in cultured cardiac mesoderm of the axolotl (Parker *et al*, 1991; Muslin & Williams, 1991). Therefore, unlike its effect on other muscle cell types, the effect of TGF-β in cardiac muscle does not seem to be growth inhibitory, but rather, differentiation inducing.

Later on in the development of the heart, TGF-β1 and TGF-β2 may interact to form the cushion tissue (Dickson *et al*, 1993). It is postulated that by inducing changes in the ECM, TGF-β1 enables the endothelial cells to migrate into the cardiac jelly (Runyan & Markwald, 1983), while TGF-β2 diffuses from the myocardium into the jelly and induces an endothelial-mesenchymal transition. Studies have shown that there is a signal emanating from the myocardium adjacent to the cardiac jelly, and that it has inductive capabilities, but whether TGF-β2 is a component of this signal is not yet known (Krug *et al*, 1985; Mjaatvedt & Markwald, 1989; Potts & Runyan, 1989; Lyons *et al*, 1990; Millan *et al*, 1991).

Continued expression of the TGF-βs in the developing heart appears to be limited. TGF-β2 and TGF-β3 are not expressed after days 17.5, and 16.5 dpc respectively, whereas TGF-β1 expression is restricted to the endothelial cells overlying the cardiac cushions until 7 days after birth (Pelton *et al*, 1991; Millan *et al*, 1991). However, in the adult heart, expression is switched back on in the myocardium (Roberts *et al*, 1981), and is up-regulated in response to mechanical load, infarction, and adrenergic agonists (Thompson *et al*, 1988, 1989; Bhambi & Eghbali, 1991). A role for TGF-β in the adult heart is supported by the finding that TGF-β can control the expression of at least six cardiac restricted genes, and can also convert isolated adult rat cardiac fibroblasts into cells with a cardiac myocyte phenotype which retain the ability to proliferate (Parker *et al*, 1990a,b; Eghbali *et al*, 1991). Therefore, from *in vitro*, and expression studies, it appears that in the adult, the TGF-βs are involved in differentiation and disease of the heart (Parker & Schneider, 1991).

1.4.6 Epithelium.

Most epithelial cells appear to be growth inhibited by TGF-β. *In vitro,* all three TGF-β isoforms inhibit the proliferation of keratinocytes, intestinal epithelial cells, prostate epithelial cells, mammary duct epithelial cells, and renal proximal tubular cells (Fine *et al*, 1985; Moses *et al*, 1985; Reiss & Sartorelli, 1987; Kurokowa *et al*, 1987; Silberstein & Daniel, 1987; McKeehan & Adams, 1988). TGF-β can also induce differentiation of certain epithelial cells (Jetten *et al*, 1986; Masui *et al*, 1986).

In vivo studies support the growth inhibitory and differentiation inducing effects of TGF-β on epithelial cells. An inverse correlation between the number of dividing cells and expression of TGF-β1 mRNA in intestinal enterocytes was found in a study by Barnard *et al* (1989), while another group found that mammary growth and morphogenesis was inhibited by the release of TGF-β from pellets (Silberstein & Daniel, 1987). Transgenic mice have also been utililised to study the effect of TGF-β on epithelial cells. Mice expressing active TGF-β under the control of the mouse mammary tumour virus exhibited hypoplasia of the mammary duct tree, whilst inhibition of cell proliferation was also observed in the skin of K1-TGF-β1 transgenic mice (Pierce Jr *et al*, 1993; Sellheyer *et al*, 1993). The increase in epidermal cell proliferation observed in the TGF-$\beta 1^{-/-}$ mice also supports the inhibitory role of TGF-β1 (Glick *et al*, 1993). There are however, exceptions to the rule. An example is the increase in cell proliferation observed by BrDU incorporation when TGF-β1 was overexpressed in the suprabasal keratinocytes in the skin of transgenic mice under the direction of the K10 promoter (Cui *et al*, 1995). This is in direct contrast with the results of Sellheyer *et al* (1993), as mentioned above, but may be as a result of the different promoters used, or the stability and level of TGF-β1 expressed (Cui *et al*, 1995).

Converse to the *in vitro* or *in vivo* data for adult epithelial cells, the expression of the TGF-βs in many embryonic epithelia is not obviously associated with growth inhibition (Millan *et al*, 1991; Pelton *et al*, 1991). TGF-β1 is expressed in the epithelia of morphogenetically active structures, such as whisker follicles, salivary glands, and tooth buds, in conjunction with TGF-β2 and/or TGF-β3 (Lehnert & Akhurst, 1988; Millan *et al*, 1991). In contrast, TGF-β1 is not expressed in the epithelia of developing sense organs, whereas TGF-β2 and TGF-β3 proteins are present (Millan *et al*, 1991; Pelton *et al*, 1991). It has been postulated that in development, TGF-β acts in an autocrine way to regulate the growth and differentiation of embryonic epithelia, and/or in a paracrine manner to induce interactions between the epithelium and underlying mesenchyme (Lehnert & Akhurst, 1988; Pelton *et al*, 1989, 1991). Recent studies investigating the distribution of the TGF-β receptors found they were localised in the mesenchyme, and absent from the epithelium, supporting the paracrine model of Lehnert & Akhurst (1988) (Lawler *et al*, 1994).

1.4.7 Haematopoiesis.

Haematopoiesis is the formation of blood. Studies using bone marrow cultures suggest that TGF-β is an inhibitor of haematopoiesis, but only has effect on certain immature cell populations (Ohta *et al*, 1987; Ottman & Pelus, 1988; Chenu *et al*, 1988). For example, murine cells induced to proliferate by IL-3, and human cells induced by IL-3 plus granulocyte/macrophage-colony stimulating factor (GM-CSF) were inhibited by TGF-β1 and TGF-β2, whereas murine cells induced by GM-CSF, or the human line stimulated by G-CSF were not (Chenu *et al*, 1988; Keller *et al*, 1988,1989; Hampson *et al*, 1988). TGF-β also effects the differentiation of certain cells in the haematopoietic lineage (Chenu *et al*, 1988). For example, the maturation of erythroid progenitor cells was reversibly inhibited when mice were given daily injections of TGF-β1 (Miller *et al*, 1992). However, these postulated activities of TGF-β1 in haematopoiesis could be completely artefactual due to the experimental conditions.

In embryogenesis, haematopoiesis begins in the yolk sac (primitive haematopoiesis), with the stem cell population initially observed at 7 days *post-coitum* (dpc) in the mouse (Moore & Metcalf, 1970). *In situ* hybridisation detects TGF-β1 at 7 dpc within the blood islands of the yolk sac (Akhurst *et al*, 1990). These blood islands contain the mesodermal haemangioblasts of the yolk sac which give rise to both endothelial and haematopoietic cell lineages, and progeny in each lineage express TGF-β1 RNA and protein (Sabin, 1920; Wilt, 1965; Miura & Wilt, 1969; Akhurst *et al*, 1990). Later in development, TGF-β1 RNA is also detected in the foetal blood cells of the liver (definitive haematopoiesis) (Heine *et al*, 1987; Akhurst *et al*, 1990).

These results suggest a role for TGF-β1 in haematopoiesis, but whether it has an effect in both primitive and definitive haematopoiesis is unclear, as there are genes which are required for only one or other haematopoietic event, for example, *c-myb*. Transgenic mice completely deficient for *c-myb* exhibit normal primitive haematopoiesis in the yolk sac, but by day 15 the embryos are severely anaemic (Mucenski *et al*, 1991). The adult-type haematopoiesis, but not the primitive haematopoiesis, is severely affected. This is also observed in *dominant white spotting* and *steel* mutants, and when either erythropoietin (EPO) or the erythropoietin receptor (EPO-R) is knocked out (Bennett *et al*, 1956; Wu *et al*, 1995). The EPO and EPO-R heterozygote embryos were normal and viable, and although the homozygote nulls exhibited a reduced primitive erythropoiesis, it was the complete block to defintive erythropoiesis which resulted in lethality at approximately day 13. In contrast, when the GATA-2 gene is disrupted, primitive haematopoiesis is affected, leading to

embryonic death at day 10-11 (Tsai *et al*, 1994). Many of these mutants appear to have abnormal haematopoiesis as a result of defective proliferation of the cells, and because the different cell types arise at different stages of development, only primitive or definitive haematopoiesis is affected.

1.4.8 Endothelium.

In vitro studies have suggested that TGF-β is inhibitory to endothelial cells. Most analyses have examined the effect of TGF-β on vascular endothelial cells, and although TGF-β1 is almost 100 times more potent than TGF-β2, both isoforms inhibit the proliferation, and migration of bovine vascular endothelial cell monolayers in culture (Heimark *et al*, 1986; Baird & Durkin, 1986; Frater-Schroder *et al*, 1986; Jennings *et al*, 1988). TGF-β1 was also found to effectively inhibit phorbol ester-induced invasion of foetal bovine heart endothelial cells into collagen (Muller *et al*, 1987). However, if the endothelial cells were seeded in the collagen gel before treatment with TGF-β1, they were not growth inhibited, but formed tube-like structures as if mimicking angiogenesis (Madri *et al*, 1988). Therefore, as vascular endothelial cells *in vivo*, like the cells in the collagen gel, are surrounded on all sides, TGF-β may actually have a role in the formation of vessels. Gene expression is also modulated by TGF-β1, with increasing expression of ECM proteins, growth factors, and protease inhibitors in capillary endothelial cells (Starksen *et al*, 1987; Daniel *et al*, 1987; Saksela *et al*, 1987; Madri *et al*, 1988).

As mentioned previously, during embryogenesis the endothelial cells of the yolk sac, which arise from the mesodermal haemangioblasts, express TGF-β1 RNA and protein (Sabin, 1920; Wilt, 1965; Miura & Wilt, 1969; Akhurst *et al*, 1990). TGF-β1 mRNA is expressed in the endothelial cells from the pro-angioblast stage through to the stage when the activated endothelial cells are forming tubes. This data, and the evidence provided in this thesis, support the idea that TGF-β1 has autocrine activity in vasculogenesis and angiogenesis.

1.4.9 TGF-β1 Knockout Mice.

In an attempt to determine the actual role of TGF-β1 *in vivo*, TGF-β1 knockout mice were generated by homologous recombination. In two independent studies, exon 6 (Shull *et al*, 1992) and exon 1 (Kulkarni *et al*, 1993) of the TGF-β1 gene were mutated *in vitro* by the insertion of a neo-cassette (Fig 3), thereby disrupting expression of the wild type protein. Chimeric mice were generated with the transgene inserted into the germ line enabling TGF-β1 knockout lines to be propagated. Both

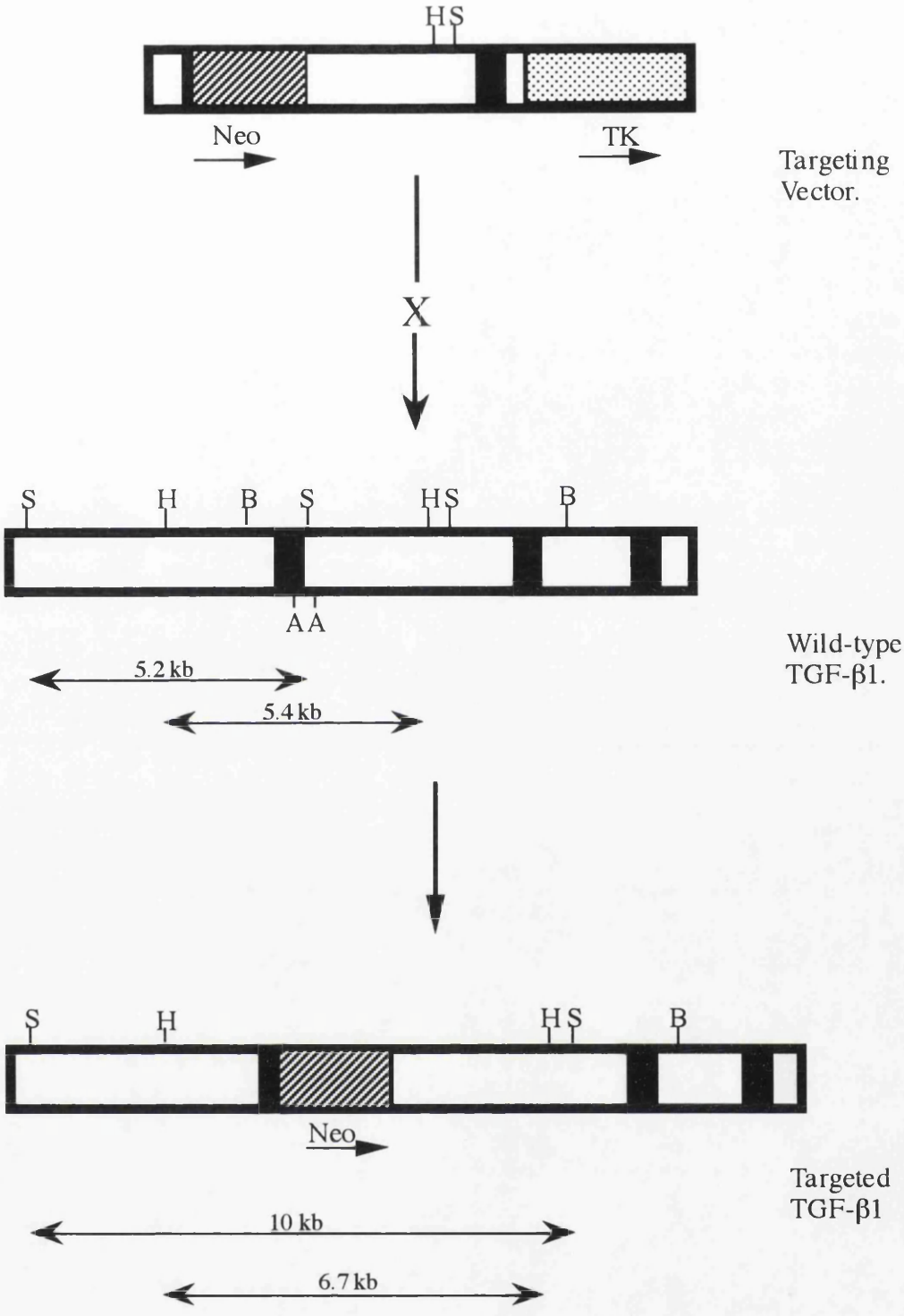


Figure 3 - Targeted Disruption of the TGF-β1 Gene by Homologous Recombination.

Diagram shows schematic representations of the targeting vector, the wild-type TGF-β1 gene, and the predicted disrupted TGF-β1 gene. Solid boxes indicate TGF-β1 exons, open boxes show introns and the promoterless region. Neo and TK (hatched boxes) when expressed confer drug resistance and sensitivity, respectively, thereby enabling selection of transformed cells (see section 1.5.1.2). A 0.56 kb sequence was deleted between *Asp* 718 (A) sites and the Neo cassette inserted. Differences in size of the *Sac* I (S) and *Hind* III (H) fragments in the wild-type and targeted alleles are shown by double-headed arrows. B - *Bgl* II.

groups found that on analysis of the transgenic offspring, mice lacking any functional TGF-β1 protein were born and appeared overtly normal. They were indistinguishable from their wild-type (TGF-$\beta1^{+/+}$) or heterozygote (TGF-$\beta1^{+/-}$) littermates. This was very unexpected after the TGF-β1 expression pattern analyses, and the results of *in vitro* and *in vivo* studies, as mentioned in the sections above. However, at approximately three weeks of age, all of the TGF-$\beta1^{-/-}$ mice exhibited severe wasting and died.

Anatomical analysis of the TGF-$\beta1^{-/-}$ mice revealed that there were no gross phenotypic abnormalities, although the TGF-$\beta1^{-/-}$ mice with exon 6 mutated did have smaller spleens and a reduced number of Peyer's patches in comparison with their normal littermates (Shull *et al*, 1992). However, on histological analysis, it was revealed that the wasting syndrome was accompanied by massive infiltration of inflammatory cell types, such as macrophages and lymphocytes, into multiple organs, and tissue necrosis, leading to organ failure, and death. The main organs affected were the heart, lungs, stomach, liver, salivary gland, pancreas, and striated muscle. Therefore, a role for TGF-β1 in the immune system was the only action supported by the depletion of TGF-β1 in the transgenic knockout mice. The immune phenotypes observed were similar to those found in autoimmune or graft versus host diseases, suggesting that TGF-β was involved in homeostasis of the cells in the immune system.

To investigate whether death of the TGF-$\beta1^{-/-}$ mice was as a result of an autoimmune response, the expression of major histocompatibility genes (MHC) was examined (Geiser *et al*, 1993). MHC Type II are normally expressed on a limited number of cell types which present antigen, enabling these cells to be identified by CD4+ T cells, which remove the "suicide" cell plus antigen by phagocytosis. However, in autoimmune diseases, MHC Type II are expressed on cells which normally do not express these molecules, and are not presenting any antigens. The CD4+ T cells will remove any cell expressing these MHC molecules. Therefore, the body is essentially fighting against, and removing healthy, normal cells. On analysis of the infiltrated TGF-$\beta1^{-/-}$ tissues, it was found that there was an increase in MHC Type II expression in these tissues in comparison to TGF-$\beta1^{+/+}$ or TGF-$\beta1^{+/-}$ littermates. This was observed prior to the inflammatory response, suggesting a correlation between the increase in MHC Type II expression and activation of the immune system. It appears therefore, that TGF-β1 may also regulate the normal expression pattern of MHC Type II in the immune system.

The effect of depletion of TGF-β1 in embryogenesis was not examined in any of the above studies, but was the subject matter of this project (section 1.8.2).

Although the phenotypes in the TGF-$\beta1^{-/-}$ mice were not as predicted from developmental studies, the results do not mean that the postulated roles for the protein, gained from *in vitro* and *in vivo* studies, are incorrect. There are numerous other proteins*in vivo*, such as the other members of the TGF-β superfamily, which may interfere, and rescue some of the effects of depletion of TGF-β1. However, by utilising additional investigative methods, the role of other proteins in the TGF-β1 knockouts could be examined. Some of these methods are discussed in the following section.

1.5 Methodological Approaches to Dissection of Gene Function During Embryogenesis.

A number of approaches may be taken to examine gene function in embryogenesis. There are several technological approaches to the manipulation of gene expression which will be reviewed below. Complementing these technological approaches, post-implantation embryo culture systems have been developed. *In vitro* culture enables the phenotype of an embryo to be examined over time as the embryo develops outside the uterus. Together these two approaches provide powerful tools with which to examine genetic manipulation and its consequences in embryogenesis.

1.5.1 Gene Knockout Methodologies.

One way to assess the function of a protein *in vivo* is to prevent its expression, essentially knocking out its function, and then examine the phenotypic result. There are several methods available to selectively knockout protein expression; antisense oligodeoxynucleotides, dominant-negative mutations, or gene knockout by homologous recombination.

1.5.1.1 Antisense Oligonucleotides (ODN's).

Antisense ODNs have been used to inhibit gene expression in a wide range of studies, mainly *in vitro* (Zamecnik & Stephenson, 1978; Miller *et al*, 1985; Matsukura *et al*, 1987; Burch & Mahan, 1991). Short sequences of single-stranded DNA (16-27 nucleotides), complementary to the mRNA of choice, are applied intracellularly (by microinjection) or extracellularly (in the medium) to the cells (Hélène & Toulme,

1990; Crooke, 1992). Theoretically, the ODNs enter the cell and bind with the mRNA forming a DNA-RNA duplex. Translation of the RNA is then prevented if the DNA-RNA duplex has formed over the ATG region thereby obstructing the translational apparatus by steric hindrance, and/or the duplex is degraded by RNAse H (Eder *et al*, 1993).

As well as being specific for a mRNA sequence, functional antisense ODNs must be stable to nucleases in order to reach the target. They must also be soluble, but still able to pass through the cell membrane (Stein & Cohen, 1988, for review). Normal ODNs were found to be extremely sensitive to nucleases, and so modified ODNs such as methylphosphonates, and phosphorothioates were devised which have been shown to have a prolonged half-life within the cell (Miller *et al*, 1985; Matsukura *et al*, 1987).

Antisense ODNs have been used to investigate the function of proteins in early development, the role of growth factors, and ECM molecules, and as potential therapeutic agents to fight disease (Matsukura *et al*, 1987; Agrawal *et al*, 1989, 1991; Woolf *et al*, 1990; Burch & Mahan, 1991; Potts *et al*, 1991; Heasman *et al*, 1992; Lallier & Bronner-Fraser, 1993). However, there are several problems with the use of antisense ODNs (Albert & Morris, 1994). Sufficient penetrance of the ODN is obtained if it is applied to cells in culture, or to surface structures, but for internal structures the number of cells affected by the ODN will be limited. Injection of the ODN into the internal tissue or organ will overcome this problem to a degree, but may result in leakage, or damage to the tissue, and is therefore not particularly efficient. If the application of the ODN is successful, the effects are usually transient, only observed within 24-48 hours. To overcome either of these problems a high concentration of ODN may be applied, and over a longer period of time, but this can be toxic. However, if too little is used, then there is no effect.

DNA-RNA hybridisation may also be problematic due to unpredicted mRNA secondary structures. To overcome this, an excess of ODN is applied (several hundred fold), which, in turn may be toxic, or promote cross hybridisation to related sequences. However, by directing the ODN at nonconserved, nonrepetitive regions of the mRNA, for example, 5' or 3' untranslated regions, the specificity is increased.

Quantitative analysis of the target protein by binding or immunoblot assays needs to be performed to assess the success of the knockout, while the specificity of the ODN can be measured by investigating the levels of related, or associated proteins. While analysing target protein levels, the turnover rate also needs to be taken into

consideration as although there may be a 100% block to new protein formation, protein formed before the application of the ODN may still remain. This "old" protein would also affect acute, transient antisense experiments, such as ODN injection.

Therefore, although the ease of application of antisense ODN's is appealling, all of these problems need to be taken into consideration before choosing this method. They also need to be noted when analysing the results from antisense ODN experiments.

1.5.1.2 Dominant Negative Mutations.

Dominant negative gene mutations result in a non-functional protein product which then competes with, and inhibits the function of the wild type protein (Herskowitz, 1987). Naturally occuring dominant negative mutations include the thyroid hormone receptor α2 (TRα2), an endogenous mutant of TRα1, and the *v-erbA* oncogene, a mutant of *c-erbA* (Katz & Lazar, 1993; Zenke *et al*, 1990). However, it is now thought possible to create dominant negative mutations in any gene, especially those whose product requires dimerisation, and thereby attempt to reveal the nature of the wild-type protein. The cDNA is mutated *in vitro*, transfected into cells, and then expressed in excess so that the translated mutant protein suppresses the action of the wild type protein.

One example of a synthesised dominant negative mutation is the retinoic acid receptor type-alpha (RARα). Although RARα is expressed in almost all haematopoietic cell lineages, its function is unknown (Gallagher *et al*, 1989). When a non-functional dominant negative mutation of RARα was transfected into the multipotent interleukin-3-dependent FDCP mix A4 murine haematopoietic cell line, basophils and mast cells developed, instead of neutrophils and monocytes (Tsai *et al*, 1992). This suggested that RARα promotes the differentiation of neutrophils and monocytes, but suppresses basophil and mast cell development.

Proteins which interact with others to exert an effect are very accessible to investigation by dominant negative mutation analysis (Herskowitz, 1987). When a dominant negative mutation was created in the A subunit of platelet derived growth factor, the activity of the protein dimer was inhibited (Mercola *et al*, 1990). Fibroblast growth factor receptor, and transforming growth factor type-beta receptor type II have also been examined by this method (Amaya *et al*, 1991; Brand *et al*, 1993). The potential for dominant negative mutational analysis is widespread, from *in vitro* studies on cells, tissues or organs, to the generation of transgenic mice to investigate the role of a protein *in vivo* (Pratt *et al*, 1990; Amaya *et al*, 1991; Brand *et al*, 1993).

As for most methods, there are advantages and disadvantages in the use of dominant-negative mutations. One of the major advantages is that the mutated cDNA can be used to create transgenic mice. The construct can also contain a sequence that directs the expression of the gene in a specific tissue type only. For example, a mouse mammary tumour virus would only be switched on in the mammary gland, and therefore drive the expression of the mutated cDNA at that site.

Nevertheless, whether the mutation is transfected into cells in culture, embryos in culture, or expressed in transgenic mice, there are several drawbacks. The effectiveness of the mutant protein may be questionable. It is possible that the mutant protein will not be able to compete with the wild-type protein, with the result that the wild-type protein will continue to perform its function. However, even if the mutant protein is functional, another difficulty is whether its expression level is high enough for dominance. Associated with this is the fact that there is always a degree of "leakage" with dominant-negative mutations, because the wild-type protein is not removed. Therefore, any result gained from a dominant negative mutation may not reveal the full and entire nature of the wild-type protein.

1.5.1.3 Generation of Knockout Mice by Homologous Recombination.

Transgenic animal production is defined by the physical integration of a segment of exogenous DNA into the genome of the germ line for transmission to the next generation (Sigmund, 1993). The DNA may be an entire cloned gene, a cDNA, or a region of a gene that has been mutated *in vitro*. There are a number of methods which can be used to deliver the DNA; microinjection into the fertilised egg, gene transfer into embryonic stem (ES) cells, or using retroviruses. Microinjection is the method normally used to "add" an exogenous gene in order to increase the expression of the protein, whereas ES cell methods are generally used to delete or "knockout" an endogenous gene. All of these methods have advantages and disadvantages, but since this project involved the use of transgenic mice generated by the ES cell method, only this system will be described in detail.

ES cells are derived from the inner cell mass of a blastocyst, can reintegrate into the inner cell mass of another blastocyst, and are totipotent. Under certain culture conditions ES cells do not differentiate thereby retaining the ability to become any cell in the entire embryo, and while in culture, modified DNA can be transfected or infected into the cells. One of the advantages of this system is that the DNA can be targeted to a specific site and disrupt a specific gene in the host genome by homologous recombination. Although there is a high incidence of random integration,

homologous recombinants can be identified by double selection with drug resistance genes, such as neomycin and gancyclovir (see below). The genetically modified ES cell is then injected into the inner cell mass of a blastocyst before implantation into a host mother, resulting in chimeric offspring. If the chimeras contain the mutation in their germ cells, the mutation can be transmitted to the next generation, and the phenotype of heterozygote or homozygote offspring should provide evidence for the function of the protein *in vivo* (Sigmund, 1993).

The ES cell method is now widely used to create transgenic mice. There are knockouts for growth factors, receptors, cell-cell adhesion molecules, cell-ECM adhesion molecules, and ECM proteins, among others. However, the results obtained with transgenic knockouts are not always as expected. Whereas the phenotypes of the IGF-II, α5-integrin, and fibronectin knockouts did agree with the postulated role of the protein (DeChiara *et al*, 1990; Yang *et al*, 1993; George *et al*, 1993), the phenotypes obtained for tenascin, *c-src*, the activins, and TGF-β1 knockouts did not (Soriano *et al*, 1991; Saga *et al*, 1992; Shull *et al*, 1992; Kulkarni *et al*, 1993; Vassalli *et al*, 1994; Matzuk *et al*, 1995a). These latter proteins were thought to be critical for embryonic development, but resulted in the birth of phenotypically normal mice of all genotypes. It is possible that these proteins are no longer required due to evolutionary change, or there may be functional redundancy with like proteins in the knockout embryos (Erickson, 1993). However, this surprising result shows that the results obtained from *in vitro* and expression studies should not be taken as indicative of function.

The greatest advantage of using ES cells and homologous recombination to create a transgenic mouse, is the ability to specifically mutate the gene of choice. However, as mentioned above, homologous recombination does not always occur, with random integration a more common event. It is now possible though, to identify homologous recombinants (Sigmund, 1993). Firstly, a neo cassette is included in the targeted cDNA, providing neomycin resistance to any cell with transgene integration and expression. To differentiate between random and homologous recombination events, the herpes simplex virus thymidine kinase (HSV-TK) gene is placed at one or both ends of the targeted cDNA construct. When the HSV-TK is expressed, the cell is rendered sensitive to the drug, gancyclovir. However, the HSV-TK is removed if the cDNA integrates to its homologous site. Therefore, only homologous integrations will survive exposure to gancyclovir. The inclusion of both the neo cassette and HSV-TK in the cDNA enables a greater percentage of the rare homologous recombinants to be identified.

One other benefit of creating transgenic animals is that the observed phenotype is likely to be a result of the transgene, rather than artefactual conditions as can occur *in vitro*. However, as mentioned previously, there are many other proteins *in vivo*, one or more of which may mask the phenotype resulting from depletion of the protein of interest. But by creating transgenic lines for each of the related proteins, and then mating them, double or multiple knockout mice can be made. Without any of the proteins which perform the same role, the knockout phenotype would almost certainly be observed.

The major disadvantage of transgenic knockout technology is that if a gene has been silenced by the insertion of a transgene, the endogenous protein is removed from that animal forever. However, attempts have been made to design methods which would enable the expression of a transgene to be controlled, using both the cre and FLP recombinases (Fig.4) (Kilby *et al*, 1993, for review).

cre is the recombinase of the bacteriophage P1, and recognises a site called *loxP*. FLP is from a yeast plasmid, and recognises FRT sites. Conventional homologous recombination techniques are used to insert a transgene containing *lox* or FRT sites into ES cells, which are then used to create transgenic mice. Another transgenic line is generated which expresses cre or FLP. Once the cre and *lox*, or FLP and FRT lines are mated, activation of the recombinase results in excision of the segment of cDNA between the recombinase recognition sites (Fig.4). This method can be used to switch genes on or off. If the sites are at either end of the cDNA, excision by cre or FLT would switch off the transgene as the whole construct is removed (Fig.4). However, the FRT or *lox* sequences can be within the transgene, with the result that excision only removes a section of the construct. This arrangement enables transgenes to be switched on (Fig.4). For example, cre mice were mated with a line carrying a lens-specific αA-crystallin promoter followed by a stop codon, then a dormant SV40 T antigen coding sequence (Lakso *et al*, 1992). The stop codon was flanked by *lox* sites, and after excision of the stop region by cre, the dormant T-antigen was activated, resulting in the formation of lens tumours in the doubly transgenic animals. So far, the recombinases appear to have great potential in transgenesis, but they need to be investigated further, and the methods refined.

Once the transgenic lines are generated, it is also possible to examine the transgenic embryos, and determine whether there are any developmental abnormalities. By using *in vitro* culture of the post-implantation embryos, the onset of these defects can be observed outside the uterus.

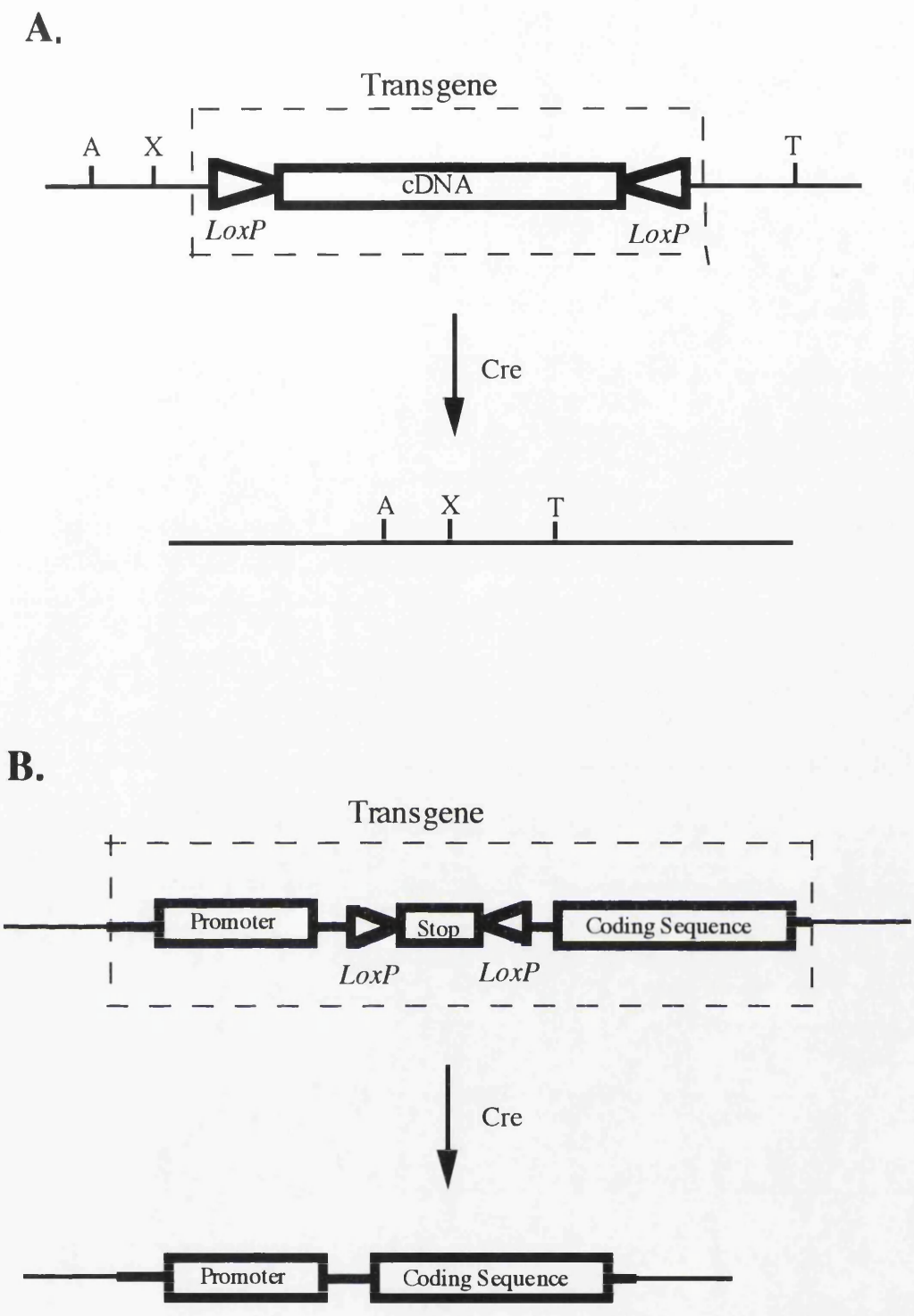


Figure 4 - The use of Cre-LoxP as a Control Switch in Transgenic Mice.

Cre is a recombinase found in the bacteriophage P1, and recognises the site *LoxP*. By mating mice transgenic for cre with a line containing the *LoxP* sequences, this system can be used to control the expression of a transgene in a transgenic mouse. The *LoxP* sequences are contained within the transgene. A) shows how cre can be used to switch off the expression of a transgene when the *LoxP* sites at either end of the cDNA. The whole transgene is removed. In B), the coding sequence is expressed after the removal of the stop codon by cre.

A, X, and T are random sites in the genome.

The FLT-FRT system can be used in place of cre-*LoxP*. FRT is the recombinase, and recognises the site FRT.

1.5.2 Post-Implantation Embryo Culture.

In the past, the major problem with studying embryonic development was that most vertebrate embryos were hidden away, either inside the uterus or a shell. Therefore, there was a great need to develop *in vitro* embryo culture systems to examine and follow developmental processes as they occurred. The first successful *in vitro* methods were devised in the sixties in response to the requirement to study the effects of drugs, such as thalidomide, on the embryo and foetus (New, 1978, for review). Since that time, embryo culture has been used to study a wide range of events including teratogenesis, specific developmental processes, such as NT closure or mesoderm formation, and the role of certain proteins in development, either by adding to the culture, or via knockout methodology (Tam & Beddington, 1987; Pratten *et al*, 1988; Gressens *et al*, 1993; Augustine *et al*, 1993; Guest *et al*, 1993; Smoak, 1993; Abbott *et al*, 1994).

8.5 to 12.5 dpc mouse embryos are most amenable to *in vitro* culture, enabling the period of major organogenesis to be studied. The culture medium initially used was 100% rat serum, which is still used today (Steele & New, 1974; New *et al*, 1976; Pratten *et al*, 1988; Walsh *et al*, 1993). However, to support normal development the serum must be immediately centrifuged, and heat-inactivated (Steele & New, 1974; New *et al*, 1976). Immediately centrifuged serum diluted with a chemically defined medium, or with saline can also be used, but as yet a fully defined medium able to support *in vitro* development is not available (Tam & Beddington, 1987; Savatier *et al*, 1990; Shepard *et al*, 1993; Guest *et al*, 1993; Smoak, 1993; Augustine *et al*, 1993; Akazawa *et al*, 1994).
Steps have been taken to determine the crucial components of rat serum, which are absent from chemically defined media. By adding various energy sources, vitamins, and amino acids to extensively dialysed serum, it was found that glucose, and numerous vitamins, including pantothenic acid, riboflavin, i-inositol, and folic acid are essential for normal development (Tanimura & Shepard, 1970; Cockroft, 1979; Sanyal, 1980; Akazawa *et al*, 1994). Analysis of serum during the culture period shows that glucose and proteins, such as α2-macroglobulin and transferrin are depleted, indicating that the embryo is utilising these factors (Sanyal, 1980; Priscott *et al*, 1983). Interestingly, amino acids do not appear to be required in the medium (Cockroft, 1979). The embryo may obtain amino acids from serum protein breakdown in the yolk sac (Freeman *et al*, 1981).

There are several other requirements for successful *in vitro* embryo culture. The most suitable oxygen (O_2) tension varies with the developmental stage of the embryo, and

should be regulated as excessive O_2 can be detrimental to the culturing of embryos (Morriss & New, 1979). 5% O_2 is required for normal neural tube closure in early somite stage embryos, and should be increased to 20% for 10-15 somite embryos, 40% for 20-30 somite embryos, and 95% for 30 somites and above (New *et al*, 1976; Cockroft, 1991). The temperature is also important and should be a constant 37-37.5°C as heat shock results in embryonic cell death and severe developmental defects (Walsh *et al*, 1993). Normal *in vitro* development can last for up to 72 hours if the medium is gassed every 12-16 hours, and changed after 24 hours, and the gas mixture is of the correct combination for the embryonic age.

Using the post-implantation embryo culture system in conjunction with the gene knockout technologies enables any effect to be observed directly. Although embryos removed from transgenic mice will already be genetically affected in the uterus, for antisense ODNs, and dominant negative mutations, the culture system can be used as an application system. By adding the ODN or cDNA to the culture medium, and culturing the embryos for 24-72 hours, at least a percentage of the cells would be affected. However, the embryos could also be injected before culture to allow any internal structures to be exposed to the cDNA or antisense ODN. By either method, any transient, or acute effect would be observed as it occurred. Culture of transgenic embryos made by homologous recombination enables the progression of a developmental defect to be followed. Removing the embryo from the uterus also allows its environment to be controlled by the investigator. In the uterus, the embryo receives many proteins, including nutrients, hormones, and growth factors from the mother, any of which may affect the knockout phenotype. In this project, the culture system was adapted to study the developmental defects in embryos from transgenic TGF-β1 knockout mice, as described in the next section.

1.6 Project Aims.

1.6.1 Development of a Culture System Lacking Transforming Growth Factors Type-beta.

A major aim of the project was to investigate the role of TGF-β1 in development by interfering with the function of this ligand/receptor system in culture utilising molecular biological techniques. At the start of the project, there was no molecular information on TGF-β receptors, thus the ligands, TGF-β1, 2, or 3, had to be the target for the disruption of TGF-β function.

The first high level gene expression of TGF-β1 and TGF-β2 is reported to be 7-8 days post-coitum (Lehnert & Akhurst, 1988; Millan *et al*, 1991). It therefore seemed pertinent to examine embryos over this period of embryogenesis if addressing the function of these genes in development. Culture of mouse and rat embryos over the period equivalent to 7-9.5 dpc in the mouse has been widely documented. However, in all previous studies, rat serum, which contains large quantities of TGF-β1, has been a major component of the culture medium (50-100%) (Steele & New, 1974; New *et al*, 1976; Tam & Beddington, 1987; Pratten *et al*, 1988; Savatier *et al*, 1990; Walsh *et al*, 1993; Shepard *et al*, 1993). Soluble ligand might act to "rescue" TGF-β-deficient embryos (Letterio *et al*, 1994). Consequently, to analyse the effects of TGF-β deficiency on embryos in culture, whether using an antisense or transgenic approach, a culture medium containing no exogenous TGF-β was required. A major part of the project was therefore devoted to establishing a culture system either depleted of serum or depleted of the TGF-β therein.

1.6.2 Analysis of TGF-β1 Deficient Embryos.

The first approach taken to deplete TGF-β in the embryo was the use of antisense oligodeoxynucleotides to selectively disrupt the translation of TGF-β mRNA. It soon became apparent that the disadvantages of this system outweighed the effort required to gain meaningful results. Moreover, during the course of the project, TGF-β1 knockout mice, generated by homologous recombination, became available *via* a collaboration with Dr A Kulkarni and Dr S Karlsson at the NIH, USA (Kulkarni *et al*, 1993).

To generate the TGF-β1 knockout mice that were used in this study, exon 1 of the TGF-β1 gene was mutated *in vitro* by the insertion of a neo-cassette, thereby disrupting expression of the wild type protein (Fig 3) (Kulkarni *et al*, 1993). Although there were TGF-$\beta1^{-/-}$ offspring, genotypic analysis showed that only 50% of the TGF-$\beta1^{-/-}$, and 75% of the TGF-$\beta1^{+/-}$ embryos were surviving gestation, and all of the others were dying *in utero* or early post-natally. This project was undertaken to determine the cause of death of the 50% TGF-$\beta^{-/-}$ and 25% TGF-$\beta^{+/-}$ embryos, and to examine why a percentage managed to survive. Letterio *et al* (1994) postulated that the surviving embryos were rescued by maternal TGF-β crossing the placenta. Therefore, to investigate this hypothesis, embryos from TGF-$\beta^{+/-}$ matings were removed from the uterus and cultured in a medium lacking TGF-β. An increase in the incidence of abnormal phenotypes would suggest that the embryos were indeed rescued by maternal TGF-β. Preliminary experiments were also performed to determine whether the lack of TGF-β affected the expression of other genes.

Chapter 2

MATERIALS AND METHODS

2.1 Medium Preparation.

2.1.1 Preparation of Rat Serum for Use in Post-Implantation Embryo Culture.

The method used to prepare rat serum was that described by Cockroft (1991). Male albino rats, average weight 550g, (Harlan Olac) were anaethetised in a jar containing ether. A ventral incision was made through the body cavity, the internal organs moved to one side, and the dorsal aorta exposed. A 21 gauge syringe needle was inserted, bevel side down, into the aorta, and the blood gently drawn into the syringe until the animal was completely bled. Blood was transferred into a centrifuge tube (Nunc), and spun immediately in a bench-top MSE centrifuge, at 2300 rpm for 20 minutes at room temperature. After centrifugation the blood was left on ice for up to 16 hours before the next step. The fibrin clot was squeezed using small "paddle-shaped" forceps to release any trapped serum, and removed before further centrifugation at 2300 rpm for 10 minutes. A sterile, plugged Pasteur pipette was used to pool the serum from several bleeds, which was centrifuged once more at 2300 rpm for 10 minutes to pellet any red blood cells remaining. The serum was aliquoted into 5-10ml amounts, and then heat-inactivated at 56°C for 40 minutes during which time it was gassed twice with 5% O_2, 5% CO_2, and 90% N_2 (BOC) to get rid of any ether. It was then placed at -70°C until required. Before using, the serum was passed through a 0.22μM Millipore filter unit to remove any particles formed while thawing.

COMPONENT	CONCENTRATION µg/ml	COMPONENT	CONCENTRATION µg/ml
NaCl	6,400	L-Valine	94
KCl	400	L-Serine	42
$CaCl_2$ (anhydr.)	200	Nicotinamide	4
$FeNO_3.9H2O$	0.10	Thiamine hydrochloride	4
$MgSO_4$ (anhydr.)	97.67	D-Ca pantothenate	4
$NaH_2PO_4.H_2O$	125	Riboflavine	0.40
D-Glucose	4,500	Pyridoxal hydrochloride	4
L-Lysine hydrochloride	146	Folic acid	4
L-Histidine hydrochloride	42	Choline chloride	4
L-Arginine hydrochloride	84	Inositol	7.20
L-Threonine	95	Phenol red	15
Glycine	30		
L-Valine	94		
L-Leucine	105		
L-Isoleucine	105		
L-Methionine	30		
L-Phenylalanine	66		
L-Tryptophan	16		
L-Glutamine	584		
L-Cysteine hydrochloride	62.75		

Table 1 - Components for Dulbecco's Modified Eagles Medium (DMEM).

List of components of DMEM. There are many factors common to both DMEM and FMP (see opposite), although the quantities vary greatly in some instances, eg. L-Glutamine. FMP contains more components than the DMEM, for example, bovine serum albumin, possibly accounting for the difference in survival of the 8.5 dpc embryos when cultured in DMEM : 50% dCP, and FMP : 50% dCP (see section 3.2.3).

COMPONENT	CONCENTRATION µg/ml	COMPONENT	CONCENTRATION µg/ml
NaCl	6,400	Ca lactate $5H_2O$	600
KCl	425	Ascorbic acid	150
$MgSO_2\ 7H_2O$	330	Bovine serum albumin	6,000
$NaHCO_3$	2,800	Nicotinamide	15
KH_2PO_3	113	Thiamine hydrochloride	3
Glucose	4,400	Ca pantothenate	0.15
L-Lysine hydrochloride	225	Riboflavine	0.15
L-Histidine hydrochloride	150	Pyridoxal hydrochloride	0.15
L-Arginine hydrochloride	70	Folic acid	0.15
L-Threonine	60	D-Biotin	0.15
L-Tyrosine	30	p-aminobenzoic acid	1.5
L-Valine	50	DL-α-tocopherol	0.75
L-Leucine	40	Cholin chloride	38
L-Isoleucine	23	Inositol	0.15
L-Methionine	40	Cyano-cobalamine	0.03
L-Phenylalanine	40	Phenol red	16
L-Tryptophan	30		
L-Glutamine	150		
L-Cysteine hydrochloride	135		

Table 1 - Components for Foetal Mouse Palate Medium (FMP).

FMP was initially used as a serum-free medium to culture foetal mouse palates (Shiota *et al*, 1990), and was therefore tried for culturing whole post-implantation embryos. Made on request from Gibco, BRL.

DMEM : 50% RAT SERUM	FMP : 50% RAT SERUM
DMEM : 25% RAT SERUM	FMP : 25% RAT SERUM
DMEM : 10% RAT SERUM	FMP : 10% RAT SERUM
DMEM : 50% dCP	FMP : 50% dCP
DMEM : 25% dCP	FMP : 25% dCP
DMEM : 10% dCP	FMP : 10% dCP

Table 2 - Post-Implantation Embryo Culture Media.

The table shows the different combinations of DMEM, FMP, rat serum, and dCP which were used to culture 7.5 dpc and 8.5 dpc embryos for 24 hours.

2.1.2 Preparation of Rat Plasma.

The blood was extracted from the rats as described above. To prepare plasma instead of serum, the blood was transferred into a tube containing heparin-coated beads (10 IU lithium heparin/ml blood) (Sterilin, Cat.No. 46.363.001) to prevent the blood from clotting. The blood was left on ice until several tubes were ready for centrifugation. It was then processed as for the rat serum above.

2.1.3 Preparation of dCP (doubly centrifuged plasma).

The required amount of plasma was allowed to thaw at room temperature before aliquoting into 1.5ml eppendorf tubes. This was performed under very basic sterile conditions. The samples were then microcentrifuged at 14000 rpm for 15 minutes at room temperature in an Eppendorf benchtop microcentrifuge. After centrifugation, the plasma was decanted into fresh eppendorf tubes, avoiding the diffuse pellet, and fatty layer on the top of the plasma. It was centrifuged again at 14000rpm for 15 minutes. The plasma was then pooled, avoiding any debris which precipitated during centrifugation.

Unlike the serum, the plasma was not able to be filtered through a 0.22μM Millipore filter unit, probably as a result of the clotting factors which are still present in the fluid. Plasma was therefore not filtered.

2.1.4 Preparation of Culture Medium.

Rat serum or dCP was combined with either Foetal Mouse Palate Medium (FMP) or Dulbecco's Modified Eagles Medium (DMEM), in varying concentrations on the day that it was required (Table 1, 2). 1ml of medium was prepared in 20ml polypropylene universal tubes (Nunc, Cat.No. 3-64238A), and equilibrated in a 5% CO_2, 37°C incubator for between 2-4 hours, before being used to culture a single embryo. Medium that was not used was frozen at -20°C, until the next culture. If not used for the next culture, the medium was not frozen again, but discarded.

2.1.5 Addition of TGF-β1 to the Culture Medium.

TGF-β1 protein (British Biotechnologies) was frozen in aliquots of 1μg/ml. 7.5μl of this stock was added to 1.5ml of FMP : dCP to give 5ng/ml. This solution was then diluted into the 1ml of culture medium to give 1ng, 800pg, 750pg, 500pg, 300pg,

250pg, 200pg, 150pg, 100pg, 50pg, 35pg, and 25pg/ml concentrations of TGF-β1 in an attempt to discover how much TGF-β1 could be tolerated by the culturing embryo.

2.2 Post-Implantation Embryo Culture.

2.2.1 Mouse Husbandry.

For each line, the male mice were mated with females to obtain embryos 7.5 to 9.5 days *post coitum* (dpc). Mice were housed in a conventional room (light 5am-7pm), or on a reversed light/dark cycle (dark 6am-4pm). For conventional animals, noon on the day the copulation plug was found was taken as 0.5 dpc, whereas for reversed cycle animals, midnight on the day the plug was found was taken as 0.5 dpc.

Several lines of mice were used in this study; Parkes/NIH/J/Ola, NIH/J/Ola, 129/sv/J/Ola, C57Bl/6J/Ola, and TGF-β1 knockouts (TNull). The TGF-β1 knockouts were originally generated on a 129/Sv/Ev genetic background (Kulkarni *et al*, 1993). These mice were then mated for 2 generations onto a C57Bl/6J background, then crossed once with an inbred NIH/Olac (Harlan Olac Ltd., UK). TGF-β1 knockout embryos used in this study were obtained from heterozygous x heterozygous matings ($TGF\text{-}\beta1^{+/-}$ x $TGF\text{-}\beta1^{+/-}$).

2.2.2 Collection of Embryos for Culture.

Pregnant females from timed matings were killed by cervical dislocation, and a vertical incision made through the body wall, enabling the uterus to be removed. Decidua were dissected from the uterus, placed 3-4 per dish in Embryo Transfer Freezing Medium (Gibco BRL) and visualised using an Olympus SZH stereo microscope. The embryos were removed from their decidua using sterile watchmakers forceps, being careful not to damage the yolk sac. If to be used for culture, any with an opened yolk sac were discarded. Reicherts membrane was removed, leaving the yolk sac and amnion intact. The embryos from the TNull line were washed in a fresh dish of Embryo Transfer Freezing Medium to remove any residual tissue which could have contained maternal TGF-β1, before placing into the culture medium.

2.2.3 Culture of Post-Implantation Embryos.

Embryos were cultured from 7.5 or 8.5 dpc for 24 hours in 1ml of medium which had been equilibrated in a 37°C, 5% CO_2 incubator for 2-4 hours. One embryo was cultured in each 20ml polypropylene universal (Nunc, Cat.No. 3-64238A). The universals were then placed in a 37°C roller incubator (BTC Engineering, Milton, Cambridge) for 24 hours, rotating at 60 rpm, during which time a further 1 or 2 gassings occured. To gas the medium, the tubes were placed in the 37°C, 5% CO_2 incubator with the lids unscrewed for 30-40 minutes, before returning to the roller incubator, with the lids tightly screwed back on, for the remainder of the culture period. After 24 hours the embryos were fixed in filtered 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS), rotating overnight at 4°C, before further processing.

2.2.4 Assessment of Embryos.

Embryos were assessed when removed from the culture system using the Morphological Scoring System devised by Brown and Fabro (1981) (Table 3). A series of developing organs and features of the embryo were observed, and each given a rating depending on its development. These ratings were added together to give the embryo score. This score was then compared with that of control embryos of comparative age (embryos that were taken directly from the uterus and fixed) to determine if the cultured embryo had developed as its counterpart *in utero*.

2.2.5 Preparation of Embryos For Sectioning and Staining.

Embryos were fixed for 4-24 hours in 4% PFA in PBS at 4°C with rotation. This was followed by washing in PBS for 30 minutes, 0.85% saline for 30 minutes, two 15 minute washes in 0.425% saline : 50% ethanol, and finally placed in 70% ethanol. Embryos could be stored in 70% ethanol at 4°C for a number of weeks. A Shandon Citadel Automatic Processor was used to process the embryos further. The cycle included three washes in methylated spirits for $1^1/_2$, $2^1/_2$, and $2^1/_2$ hours; a wash in 100% ethanol for 2 hours; three washes in xylene, all for $^1/_2$ an hour; and finally two washes in 60°C paraffin wax for 2, followed by 4 hours. The embryos were then embedded in paraffin wax and stored at 4°C until use.

Table 3 - The Brown-Fabro Scoring System.

The table shows the scoring system devised by Brown and Fabro (1981). The embryonic characteristics listed are analysed in each embryo, and classified into one of the description categories. Each of these categories has a score, represented by the numbers at the top of the table Once all of the characteristics are examined, the numbers are added together, resulting in the embryos total score. This figure represents the developmental stage attained by the embryo, and can be used to compare and contrast the development of different embryos.

	0	1	2	3	4	5	Total
Yolk Sac	No visible, or scattered blood islands	Corona of blood islands w or w/o anastamoses	Vitelline vessels with few yolk sac vessels.	Full yolk sac plexus of vessels	Yolk stalk obliterated, vitelline vein & artery well separated		
Allantois	Allantois free in exocoelom	Allantois fused with chorion	Umbilical vessels	Separate aortic origins of umbilical and vitelline vessels			
Flexion	Ventrally convex	Turning	Dorsally convex	Dorsally convex with spiral torsion			
Heart	Endocardial rudiment not visible, or visible but not beating	Beating "s" shaped cardiac tube	Convoluted heart tube	Bulbis cordis, atrium commune and ventriculus communis	Dividing atrium commune		
Caudal Neural Tube	Neural plate or Neural folds	Closing, but unfused neural folds (groove)	Neural folds fused at level of somites 4/5	Posterior neuropore formed, but open	Posterior neuropore closed		
Hind Brain	Neural plate	Rhombomeres A and B	Fusing folds	Completely fused	Pronounced pontine flexure with transparent roof of 4th ventricle		
Mid Brain	Neural plate	Mesencephalic brain folds	Fusing folds	Completely fused	Visible division between mesencephalon and diencephalon		
Fore Brain	Neural plate or no visible prosecephalon	Prosecephalic brain folds	Completely fused prosecephaolon	Visible telencephalic evaginations	Well elevated telencephalic hemispheres		
Otic System	No sign of otic development	Flattened or indented otic primordium	Otic pit	Otocyst	Otocyst with dorsal recess	Otocyst with endolymphatic duct	
Optic System	No sign of optic development	Sulcus opticus	Elongated optic primordium	Primary optic vesicle with open optic stalk	Indented lens plate	Lens pocket or lens vesicle	
Olfactory System	No sign of olfactory development	Olfactory plate	Olfactory plate with rim	Distinct olfactory ridges	Lateral nasal process and medial rim		
Branchial Bars	None visible	I visible	I and II visible	I, II and III visible	II overgrowing and obscuring III		
Maxillary Process	No visible separation of maxilla and bar I	Maxillary process demarcated visible cleft anterior to bar I	Maxillary process fused with nasal process				
Mandibular Process	Medial edges of bar I less than 5% fused	First branchial bars fused and forming mandibular process					
Fore limb	No sign of fore limb development	Distinct evagination of Wolfian crest at level of somites 7-11	Fore limb bud	Paddle shaped fore limb bud	Distinct apical ridge on fore limb bud		
Hind limb	No sign of hind limb development	Distinct evagination of Wolfian crest at somites 26-30	Hind limb bud	Paddle shaped hind limb bud			
Somites	0-6	7-13	14-20	21-27	28-34	35-61	

2.2.6 Preparation of Slides.

Slides were dipped in a 2% 3-Aminopropyl-triethoxysilane (TESPA, Sigma) in acetone solution for 5-10 seconds before two washes in 100% acetone, followed by two washes in Milli-Q water. They were then baked dry at 42°C before storing in boxes at room temperature.

2.2.7 Sectioning of Embryos.

Embryos embedded in paraffin wax were serially-sectioned using a microtome. 5 micron thick sections were cut and lifted onto a glass slide. A small amount of 30% ethanol was placed onto the slide and under the sections to smooth out any wrinkles. The sections were then floated onto Milli-R water at 42°C. TESPA-coated slides were placed under the floating sections and used to pick them out. The slides were then covered, without touching the sections, and left at 42°C until dry.

2.2.8 Pretreatment of Sections Before Staining.

Dewaxing and rehydration of the sections was performed before any staining procedure. Slides were placed in 20 microscope slide holders, and soaked in Histoclear™ for two 7 minute periods. This was followed by two 2 minute immersions in 100% ethanol, then a single immersion for 2 minutes in each of 90%, 70%, and 50% ethanol dilutions. The sections were then ready for staining.

2.2.9 Dehydrating and Mounting of Sections.

Following staining, the slides were dehydrated through an ethanol series (30%, 50%, 70%, 90%, 100%, and 100% for 2 minutes each), followed by two soaks in Histoclear™ the first 2 minutes, then 7 minutes. Coverslips were mounted onto the sections using 300µl of Gurr's DePeX Neutral Mounting medium (BDH, Cat.No. 361254D) per slide in a fume hood, and left to dry overnight.

2.2.10 Staining Sections for Haematoxylin and Eosin (H&E).

After the dewaxing and rehydrating process, the sections were dipped in filtered Harris' haematoxylin (BDH, Cat.No. 35128) for 10-30 seconds, rinsed under cold running water for approximately 30-60 seconds, then placed in Scott's tap water (20g $MgSO_4$, 20g $NaHCO_3$ / litre of water) for 2 minutes. Following a further rinse in tap water, the slides were stained in eosin (5g eosin, 50mls saturated acetic picric acid,

400mls water, 2.5g potassium dichromate, 50mls absolute alcohol, added in that order / 500mls stock. Diluted 1 in 4.5 with water for working concentration) for 2-5 minutes before dehydrating and mounting.

2.2.11 Injection of BromoDeoxyUridine into 9.5 dpc Embryos.

BromoDeoxyUridine (BrDU) is a substitute base for thymidine, and is incorporated into DNA during the cells DNA replication phase. By using immunohistochemistry, it is possible to identify cells which were in s-phase of the cell cycle while the BrDU was present. It was used in this study to show whether the endothelial cells of the yolk sac were proliferating faster in the homozygote null embryos than in the wild-types of the TGF-β1 knockout mice, and also to demonstrate the difference in growth rates in embryos grown in different media.

To analyse the growth rates of embryos grown in different media, 23 hours after the beginning of culture, embryos were removed from the universal into Embryo Transfer Freezing medium (Gibco BRL). Using a pulled micropipette, 1μl of 10mM BrDU (Boerhinger Mannheim) was injected into the yolk sac cavity. The embryo was then transferred back into the culture medium and the culture continued for a further hour in a 37°C roller incubator. The embryos were then fixed in 4% PFA in PBS at 4°C with rotation before further processing.

For the TGF-β1 knockout analysis, 9.5 dpc embryos from timed TGF-$\beta1^{+/-}$ x TGF-$\beta1^{+/-}$ matings were removed from the uterus and dissected in Embryo Transfer Freezing medium (Gibco, BRL), leaving the yolk sac and ectoplacental cone intact. After injection of 1μl of 10mM BrDU into the yolk sac cavity, the embryo was cultured in FMP : 50% dCP for an hour at 37°C in a roller incubator. Following the culture period, the embryos were fixed in 4% PFA in PBS, scored for phenotype, and half of the embryo was removed for genotype analysis. The rest of the embryo and yolk sac was fixed overnight at 4°C, constantly rotating, before further processing.

2.2.12 Staining for BrDU in Embryo and Yolk Sac Sections.

Embedded embryos previously injected with BrDU were sectioned, dewaxed and rehydrated before staining for the substitute base, BrDU. The slides were manipulated in several ways during the staining process. They were held in microscope slide holders to be exposed to PBS, hydrogen peroxide, Proteinase K, PBS plus sheep serum and bovine serum albumin, which were all in 250ml glass troughs. When the slides were in the troughs a very small stirring rod was used to circulate the fluid. For

the application of the primary blocker, the primary antibody, the secondary antibody, ABComplex, and 3,3'-Diaminobenzidine Tetrahydrochloride (DAB), the slides were removed from the holders and laid flat in humid trays so that the solution could be placed directly onto each section.

After rehydration the sections were washed for three 5 minute periods in PBS, followed by blocking of the endogenous peroxidase by incubating in 1.2% hydrogen peroxide (4mls 30% H_2O_2, 96mls methanol) (Sigma) for 20 minutes. After a further three 5 minute washes in PBS, the slides were bathed in Proteinase K (20μg/ml in 50mM Tris, pH8.0) (Sigma) for 15 minutes at room temperature, followed by another three 5 minute washes in PBS. Before adding the primary blocker [5ml PBS, 0.6ml 1% gelatin (BDH), 60μl 10% bovine serum albumin (BSA) (Sigma), 180μl sheep serum (Serotec)], the slides were removed from the holder and placed in a humid chamber after drying round each section with a tissue. Care was taken not to let the sections dry out. 20-50μl of the primary blocker was added to each embryo section, and left for 30 minutes at room temperature. 10μl of undiluted mouse IgG anti BromoDeoxyUridine antibody (Amersham) replaced the blocker on each section, and small pieces of coverslips laid on top. The slides were covered, and stored at 4°C overnight. The following day the slides were washed three times in PBS containing sheep serum and BSA (1ml sheep serum, 1ml BSA / litre of PBS), for 5 minutes each time. The coverslips slid off gently into the PBS. Each slide was then dried, carefully avoiding the sections, and placed in the humid tray. The pre-blocked, biotinylated secondary antibody, sheep anti-mouse antibody (Amersham), was applied, diluted 1 : 500 in the secondary blocker (4.3ml PBS, 0.5ml 1% gelatin, 50μl 10% BSA, 150μl sheep serum, 10μl antibody). 20-50μl of this solution was added to each section, and left for an hour at room temperature. The three 5 minute PBS washes were repeated once more, before the slides were dried, placed in the tray, and the sections exposed to the ABComplex (DAKO) for 30 minutes. [ABComplex was made up 30 minutes previously by adding one drop of solution A (avidin) and one of B (Biotinylated Horseradish Peroxidase) to 5mls of PBS]. A further two 5 minute washes in PBS were performed to remove the ABComplex. Back in the tray, 20-50μl of DAB (1 10mg tablet of 3,3'-Diaminobenzidine Tetrahydrochloride / 15mls PBS, filtered then added 12μl of H_2O_2) (Sigma) was added to each section for approximately 3-5 minutes until the brown stain appeared. The slides were then quickly placed in water, and rinsed under the tap to prevent over-staining. Dehydration and mounting was then carried out.

2.2.13 Production of Embryo Powder, and Its Use In Pre-Blocking Antibody.

14-14.5 dpc embryos were dissected from the uterus and decidua in cold PBS. The yolk sac remained attached to the embryo, but the ectoplacental cone was removed. Using sterile forceps, the tissue was chopped into as many small pieces as possible, before transferring into an ice-cold sterile homogeniser with as little PBS as possible. The tissues were homogenised on ice before adding 4 volumes of ice-cold acetone, and leaving on ice for 30 minutes. The homogenate was aliquoted into 1.5ml eppendorf tubes, and microcentrifuged at 14000 rpm for 10 minutes at room temperature in an Eppendorf benchtop microcentrifuge. The supernatant was removed, and the pellet washed in ice cold acetone, before spinning for several seconds at 14000 rpm to deposit the pellet at the bottom of the tube again. After removal of the acetone the pellet was tipped out and flattened as much as possible between two pieces of filter paper. This was then covered, and the embryo homogenate left to dry overnight at room temperature, before being ground to a fine powder. The embryo powder could be stored in an airtight container at 4°C indefinitely.

To block the sheep anti-mouse antibody used in the BrDU staining protocol (2.2.12), 3mg of embryo powder was added to 0.538ml of PBS, and heated at 70°C for 30 minutes before quenching on ice. To this, 5µl of sheep serum, and 1.25µl of the antibody was added, and the solution rotated at 4°C for at least an hour. The embryo powder was pelleted after microcentrifugation at 14000 rpm for 10 minutes, and the supernatant removed to a new eppendorf. 62µl of 1% gelatin, 6.25µl of 10% BSA, and 13.75µl of sheep serum was added to the supernatant, and the antibody / secondary blocker solution was ready for use.

2.2.14 Extraction of Embryonic DNA.

The embryo and its yolk sac were removed from culture and digested overnight in 100µl of lysis buffer (100mM Tris-HCl, pH8.5, 0.2% SDS, 5mM EDTA, 200mM NaCL) plus 100µg/ml proteinase K at 55°C. An equal volume of phenol (Biophenol, Camlab) was added to the digested embryo, and vortexed for 10 seconds to ensure thorough mixing. Centrifugation at 14000 rpm for 2-5 minutes at room temperature in an Eppendorf benchtop microcentrifuge separated out the organic and inorganic layers divided by the layer of protein. The top solution was removed without disturbing the protein layer, and placed into a new eppendorf. An equal volume of chloroform (Fisons) was added, and the solution vortexed for 10 seconds before

centrifugation as above. The top layer was decanted into another eppendorf and 2 volumes of ethanol plus 1/10th volume of 3M sodium acetate were mixed in. To precipitate the DNA the samples were stored at -70°C for at least one hour, followed by centrifugation at 14000 rpm for 30 minutes at 4°C. The supernatant was removed and the pellet washed twice in 70% ethanol. After air drying, the DNA was dissolved in 40µl of TE buffer (10mM Tris, pH8.0, 1mM EDTA).

2.2.15 Measuring the Protein Content of Cultured Embryos.

The Lowry Method (Lowry *et al*, 1951) was employed to measure embryonic protein content. For protein analysis, the cultured or control embryos and their yolk sacs were snap-frozen in liquid nitrogen after scoring. On thawing the tissues were dropped into 1ml of 0.5M sodium hydroxide, and digested for 2-3 hours while rotating at room temperature, with occasional pipetting to aid the digestion.

0, 10, 25, 50, 75, 100, 150, 200, 250, 300, 350, 400, 450, and 500 µg/ml protein standards were also prepared from a 1mg/ml stock of BSA in water. The correct volumes for the desired concentrations were added to 0.5ml of 1M sodium hydroxide and made up to 1ml with water. The standards were treated as for the embryonic tissues.

During the digestion the solutions required for the assay were prepared. 1g of sodium tartrate and 0.5g of cupric sulphate were dissolved separately in 50ml of water. When both were completely dissolved the two solutions were mixed together. If a precipitate formed the solution was discarded. 6mls of the sodium tartrate / cupric sulphate solution was then added to 300mls of 2% sodium carbonate in water. 5mls of this solution was then added to each of the samples and the standards, followed by the addition of 0.5ml of Folin and Ciocalteu's phenol reagent (BDH). The tubes were thoroughly shaken after each addition. 40 minutes was allowed for the colour to develop after which the absorbancy of each sample was measured twice using a Philips UV/VIS spectrophotometer at A680nm, and the amount of protein present was calculated.

2.3 Cell Culture.

2.3.1 Introduction.

CCL64 cells (mink lung epithelial cells, Mv1Lu) (European Collection of Animal Cell Cultures) were cultured in Special Liquid Medium (SLM) (Gibco BRL) supplemented with 10% foetal bovine serum (FBS), 10mM glutamine, and 1% penicillin/streptomycin (all Gibco BRL), and maintained at 37°C in $75cm^2$ tissue culture flasks (Nunc) in an atmosphere of 5% CO_2. CCL64 cells are inhibited by TGF-β1 as long as they do not become confluent, therefore when the cells neared 75% confluency, generally every 2-3 days, they were split. The medium was removed and the cell layer washed 1x in PBS (Gibco BRL), followed by 1x with verscene (0.2g KCl, 8g NaCl, 0.2g EDTA, 0.29g $NaHCO_3$, 5ml Pen./Strep., 0.5g D-Glucose / 500ml water). To detach the cells from the flask they were incubated at 37°C with 2.5mls of 1x trypsin (Trypsin EDTA in Modified Puck's saline A, Gibco BRL, Cat.No. 45300-027) for approximately 3-5 minutes, following which the trypsin was inactivated by adding 7.5mls of serum-containing medium. The cell suspension was seeded at 1 in 10, 1 in 5, or 1 in 2 dilutions with new media, and returned to the CO_2 incubator. When required, cell numbers were estimated using a haemocytometer.

2.3.2 Cryopreservation of Mammalian Cell Lines.

Cryopreservation of cells was performed at approximately 75% confluency, that is 5-$10x10^5$ cells per flask. Cells were harvested as described in 2.3.1. and the trypsin was inactivated by addition of media. The cell suspension was placed into a 20ml universal tube and centrifuged for 10 minutes at 1500 rpm in a benchtop MSE centrifuge. The supernatant was removed and the cell pellet resuspended in 1ml 10% Dimethyl Sulphoxide (DMSO, Sigma) in media. This suspension was deposited in a 1.8ml cryostat tube (Nunc), wrapped in bubblepak and placed at -70°C for 2 days to allow slow freezing.The tubes were transferred to liquid nitrogen for long term storage. When a stock was required, it was thawed at 37°C before plating out in 9mls of media in a $75cm^2$ flask. Following overnight incubation at 37°C with 5% CO_2, the medium was removed, the cells washed 2x with PBS, and fresh medium applied.

2.3.3 CCL64 Inhibition Assay.

The CCL64 assay (Danielpour *et al*, 1989) allows the measurement of active TGF-β1 in a sample by measuring the amount of tritiated thymidine incorporation by the cells. Since CCL64 cells (Mv1Lu) are inhibited by TGF-β1, if there are high levels of TGF-

β1, the cells do not proliferate, and therefore do not incorporate tritiated thymidine, whereas the reverse is true if there is no TGF-β1 in the samples. In this study, the CCL64 assay was used to determine the amount of TGF-β1 in rat serum and rat plasma.

75% confluent cell layers were washed and trypsinised before suspension in 10mls of SLM plus 10% FBS. Centrifugation at 1500 rpm for 10 minutes in a MSE bench-top centrifuge pelleted the cells, which were then resuspended in 10mls CCL64 assay medium (DMEM, 0.1% FBS, 10mM HEPES) to wash the cells and remove the high levels of FBS. (FBS contains TGF-βs and would therefore interfere with the assay.) The cells were centrifuged once more at 1500 rpm for 10 minutes before resuspension at 10^5 cells/ml in the assay medium. They were seeded at 0.5ml / $5x10^4$ cells per well into 24 well plates, and incubated at 37°C in a 5% CO_2 incubator for 1-2 hours. A titration curve was made using known quantities of TGF-β1 (British Biotechnologies, Cat.No.101-B1), from 10pg/ml-1ng/ml. The experimental samples were assayed in several dilutions, 0.5-100%, with and without the addition of 1μl of 10mg/ml TGF-β antibody (R&D Systems, Cat.No. AB-101-NA) to determine whether or not any inhibition observed was due to TGF-β1 in the sample. All serum and plasma samples were heated at 94°C for 15 minutes, before the addition of the antibody, to activate any latent TGF-β1 and to inactivate any heat-sensitive stimulatory factors present in serum and plasma. All samples were measured in triplicate. After adding the 200μl samples to each well, the plates were returned to the incubator for 22 hours. 1.5μCi of tritiated thymidine (Amersham) in 100μl of CCL64 assay medium was then added to each well, and incubated for a further 2 hours before the medium was aspirated. The cells were washed 2x with PBS, then fixed at room temperature with 0.5ml of 3:1 methanol:acetic acid for an hour. The fixed cells were washed 2x in 80% methanol before trypsinisation with 0.5ml of trypsin/verscene (50:50) for an hour at room temperature. 0.5ml of 1% SDS was added to each well and left for 5 minutes before each sample was transferred to 10mls Ecoscint A (National Diagnostics) in a scintillation vial. The amount of tritiated thymidine incorporated was measured using a Texas Instruments scintillation counter.

2.4 RNA Analysis.

2.4.1 Extraction of RNA from 1/2 Embryos and Yolk Sacs.

All tips, eppendorfs, and solutions (except those containing Tris, or organic solvents) were treated with 0.01% DEPC before sterilisation to prevent the degradation of the RNA by RNAses. A scaled-down version of the method described by Chomczynski and Sacchi (1987) was used to extract the RNA from 8.5 or 9.5 dpc transgenic TGF-β1 embryos and yolk sacs. 1/2 of the embryo was required for genotype analysis, and so only 1/2 was available for RNA extraction. The RNA was extracted separately from the yolk sac and the embryo. When dissected free of contaminating material, the tissues were placed in eppendorfs and snap-frozen in liquid nitrogen, then stored at -20°C until required. Before proceeding with RNA extraction they were thawed on ice. 550µl of solution D [4mM guanidinium thiocyanate (Fluka), 25mM sodium citrate, 0.5% sarkosyl, 0.1mM β-mercaptoethanol (Sigma)] was added to each tube and the disaggregation of the tissue aided by pipetting. 25µl 2M sodium acetate, 200µl phenol, and 55µl chloroform were added separately with gentle mixing after each reagent. Once all three solutions were added, the tubes were vortexed for 10 seconds before incubation on ice for 15 minutes. The samples were then microcentrifuged for 20 minutes at 14000 rpm at 4°C in an Eppendorf benchtop microcentrifuge. The top aqueous layer was transferred to a fresh eppendorf and 700µl of isopropanol added. The solution was mixed and placed at -20°C for at least 1 hour to precipitate the RNA. The RNA was pelleted by microcentrifugation at 14000 rpm for 20 minutes at 4°C, and the supernatant removed. The pellet was redissolved in 100µl of solution D, and an equal volume of isopropanol added before a further precipitation at -20°C for at least an hour. The tubes were centrifuged for 10 minutes at 14000 rpm at 4°C to pellet the RNA, which was then washed 2x in 70% ethanol, air-dried and resuspended in 5-10µl of DEPC-treated water. The RNA yield was not determined as there was such a small amount it was all required for experimentation.

2.4.2 RNA Gel Electrophoresis.

RNA of unknown concentration from a single 1/2 embryo or yolk sac, or from 3 1/2 embryos or yolk sacs combined, was run in a 1.5% denaturing agarose gel containing 16.6% formaldehyde and 1x MOPS (200mM MOPS Sodium salt, 50mM Sodium Acetate, 10mM EDTA, pH 7.0). 50% formamide, 2.2M formaldehyde, and 1x MOPS was added to 5µl of RNA to a total volume of 10µl, and incubated at 55-60°C for 10 minutes before quenching on ice. 1/6th volume of 10x loading buffer was added (50% glycerol, 1mM EDTA, pH8.0, 0.25% bromophenol blue, 0.25% xylene cyanol), and

the samples loaded into the wells. RNA, DNA, or ribosomal RNA ran alongside the samples and were used as size markers. Electrophoresis occurred at 50-100V for 4-8 hours in circulating 1x MOPS to prevent depletion of ions at the anode.

2.4.3 Northern Blotting.

After electrophoresis, as described above, the gel was washed for 20-30 minutes in Milli-Q water with 2-3 changes to get rid of the formaldehyde. The marker lanes were cut off and stained for 10 minutes in 1.5μg/ml ethidium bromide before destaining in Milli-Q water for 2-16 hours The markers were then viewed on a UV transilluminator and photographed alongside a scale for reference. The rest of the gel was placed on a piece of 10x SSC (20x SSC ; 3M sodium chloride, 0.3M sodium citrate) soaked 3MM Whatman paper, which was acting as a wick to a reservoir of 10x SSC. Hybond N paper was cut to the size of the gel and placed on top, and any trapped air bubbles smoothed out. Two pieces of dry 3MM Whatman paper were placed on top of the Hybond N, and the area surrounding the gel was sealed with clingfilm. This was to prevent the evaporation of the 10x SSC reservoir, and to ensure its passage only through the gel. A stack of paper towels were placed on top of the dry Whatman paper, followed by a small weight (1kg). The blot was left overnight, and the following day the RNA was fixed on to the filter by UV irradiation for 4 minutes. The filter was wrapped in clingfilm and stored at 4°C until use.

2.4.4 Random Prime Labelling of DNA.

50-100ng of insert DNA prepared as described in 2.6, was labelled with α^{32}P dCTP (Amersham) using a Boehringer Mannheim random primed DNA labelling kit. The insert DNA was combined with Milli-Q water to a total volume of 23μl and heated at 100°C for 10 minutes before quenching on ice. 1/10th total volume (4μl) of reaction buffer, 100mM (2μl) of each dNTP (except dCTP), 1 unit (2μl) Klenow enzyme, and 50μCi (5μl) of α^{32}P dCTP was added to the DNA, and the reaction incubated at 37°C for 2-4 hours. The mixture was then passed through a Nick column (Pharmacia) with 2x 400μl of TE buffer (10mM Tris, pH8.0, 1mM EDTA) to remove any unincorporated nucleotides, collecting the second 400μl of solution. Determination of the specific activity of the probe was performed using a Texas Instruments scintillation counter, and if found to be less than 10^8 cpm/μg the probe was discarded. Immediately before use, the probe was boiled at 100°C for 10 minutes to denature the DNA, then quenched on ice.

2.4.5 Northern Hybridisation.

The Hybond N filter and a fine mesh were soaked in 2x SSC before rolling together and placing in a hybridisation bottle with 10-15mls of prehybridisation buffer [5x SSC, 1x Denhardts (0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% BSA), 50% formamide, 0.5% SDS, 100μg/ml salmon sperm DNA] depending on the size of the filter. Incubation occured for 3-5 hours at 42°C. The radioactive probe was denatured and added at $3x10^7$ cpm to the hybridisation buffer (prehybridisation buffer with the addition of 10% dextran sulphate). This replaced the prehybridisation buffer, and incubation at 42°C continued for a further 16 hours.

2.4.6 Washing of Northern Blot Filters after Hybridisation.

After hybridisation, the Hybond N filter was washed for 10 minutes at 42°C with 2x SSC and 0.1% SDS in the hybridisation bottle. The filter and mesh were then placed in a box, and washed with 0.2% SSC and 0.1% SDS at 65°C for approximately 15 minutes with shaking. The counts were checked and washing continued until they reached approximately 10 cpm. The filter was exposed to Kodak X-Omat autoradiographic film plus intensifying screens, and placed at -70°C. The time period before the film was developed depended on the levels of the transcript trying to be detected, and on the intensity of the radioactive filter.

2.4.7 Reverse Transcription (RT) of RNA to cDNA.

7μl of RNA (total RNA from 1/2 an embryo, a yolk sac, or 3 1/2 embryos or yolk sacs separately combined) was incubated at 90°C for 5 minutes before quenching on ice. 1μl (50ng) of oligo d(T) was added, followed by incubation at 65°C for 10 minutes. After quenching on ice 4μl of 5x RT buffer (250mM Tris HCl, pH8.3, 375mM potassium chloride, 15mM magnesium chloride), 5μl of 2.5mM dNTPs, 1μl 0.1M DTT, 1.2μl of Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT) (Gibco BRL), and 0.8μl of RNA Guard(RNAse inhibitor)were added and the reaction incubated at 37°C for 1 hour. The reaction was stopped by heating at 95°C for 5 minutes, then quenched on ice. The mixture was stored at -20°C until required. As for the RNA from 1/2 embryos or single yolk sacs, the yield of cDNA was not measured.

2.4.8 Reverse -Transcription Polymerase Chain Reaction (RT PCR).

4µl of the 20µl cDNA mixture resulting from reverse transcription was combined with 15mM magnesium chloride, 1x buffer (Cambio), 50pmoles of the specific sense and antisense primers (Table 4), 330mM dNTPs, and 1 unit of Taq polymerase (Cambio) in a total volume of 30µl. A layer of mineral oil was added to prevent evaporation and the reaction mixture placed in a DNA Thermocycler (Perkin Elmer Cetus). Each PCR reaction consisted of the following sequential steps : denaturation at 94°C for 1 minute, annealing at 61°C for 1.5 minutes, and elongation at 72°C for 2 minutes. There were 33 amplification cycles, finally terminating with a 5 minute incubation step at 72°C. The PCR products were stored at 4°C until analysed by gel electrophoresis on a 1.5% agarose gel.

2.5 Genotyping of TGF-β1 Transgenic Mouse DNA.

2.5.1 Extraction of Mouse Tail DNA.

0.5cm of the tail of an anaesthetised mouse was removed using a hot, sterile scalpel blade to ensure cauterisation. The tail tip was placed into 700µl of tail lysis buffer (100mM Tris, 0.2% SDS, 5mM EDTA, 200mM sodium chloride / 500mls) containing 100µg/ml proteinase K. Digestion occurred overnight at 55°C, and the tubes microcentrifuged at 14000 rpm for 10 minutes at room temperature in an Eppendorf benchtop microcentrifuge to pellet the tail debris. The supernatant was decanted into a fresh 1.5 eppendorf, and an equal volume of isopropanol added to precipitate out the DNA on shaking. Using the end of a sterile yellow tip, the DNA was lifted out and placed into another fresh eppendorf. After air drying the DNA was resuspended in 100µl of 1x TE.

The transgenic embryo DNA was extracted as described in 2.2.14.

2.5.2 Genotyping by Polymerase Chain Reaction (PCR).

Tail DNA and embryo DNA was genotyped by the same method, the only difference was the amount of DNA added per reaction. 1µl of tail DNA was enough to produce a result, whereas 3-5µl of embryonic DNA was required. The DNA was subjected to a denaturation step of 15 minutes at 94°C before adding to the reaction mix. The reaction mixture consisted of 15mM potassium chloride, 1x buffer (Cambio), 50pmoles of the specific sense and antisense primers (Table 4), 330mM dNTPs, and 1

A. Primers for Genotyping TGF-β1 Transgenic Offspring.		
No.95	5'	GAG ATG AAG CCC ACT AGA G 3'
No.96	5'	CGT GCG CCT GTC GCT TTC TG 3'
No.97	5'	GCG GAC TAC TAT GCT AAA G 3'
No.98	5'	GGT CAC CCG CGT GCT AAT GG 3'
TGFO	5'	AGG GAG CTG GTG AAA CGG AA 3'
PGK-1	5'	TCC ATC TGC ACG AGA CTA GT 3'
B. Primers to Amplify TGF-β2.		
β2 5'	5'	AAA GCC AGA GTG GCC GAG CAG 3'
β3 3'	5'	CAC TTC GCT TTT ATT CGG GAT 3'
C. Primers to Amplify TGF-β3.		
β3 5'	5'	TGA GCA AGC TCA GGC TCA CCA 3'
β3 3'	5'	TAG CGC TGC TTG GCT ATG TGC 3'
D. Primers to Amplify GAPDH.		
GDH 5'	5'	TGA GTA TGT CGT GGA GTC TAC 3'
GDH 3'	5'	GGC CAT GTA GGC CAT GAG GTC 3'
E. Primers to Amplify *Flk-1*.		
Flk5'	5'	CAA CAC AGC AGG AAT CAG TC 3'
Flk3'	5'	AAT CTA AGC AGC ACC TCT CTC 3'

Table 4 - Oligonucleotides for PCR.

Shown above are the primers required to amplify the normal, and null TGF-β1 alleles by PCR (A). Primers no.95-98 were used in the same tube to amplify the normal TGF-β1 allele from embryo or tail DNA by nested PCR, as shown in Fig.3. Primers TGF0 and PGK-1 amplified the null allele in another eppendorf tube, with a separate, but identical DNA sample. The primers used to amplify TGF-β2 (B), TGF-β3 (C), GAPDH (D), and *Flk-1* (E) by RT-PCR are also shown.
All sequences are written 5'-3'.

unit of Taq polymerase (Cambio) in a total volume of 30µl. Nested primers were used to identify the normal allele (Fig.3). Two were in the deleted portion of the first exon, and the other two in the first intron. The amplification product for the normal allele was approximately 625bp. To identify the null allele, one primer was located in the first exon, and the second in the Neo cassette, the resulting PCR product being approximately 375bp. Before starting the amplification in a DNA Thermocycler (Perkin Elmer Cetus), a layer of mineral oil was placed on top of the reaction mixture to prevent evaporation. Each PCR cycle consisted of the following sequential steps : denaturation at 94°C for 1 minute, annealing at 61°C for 1.5 minutes, and elongation at 72°C for 2 minutes. There were 30-35 amplification cycles, finally terminating with a 5 minute incubation step at 72°C. The PCR products were stored at 4°C until analysed by gel electrophoresis on a 1.5% agarose gel.

2.5.3 Analysis of PCR Products.

The products of PCR were stored at 4°C until analysed. 10µl of the product was mixed with 1µl of 10x DNA gel loading dye (0.25% bromophenol blue, 25% ficoll), and loaded into the wells of a 1.5% agarose gel containing 1.5µg/ml ethidium bromide. A 1kb DNA ladder was also loaded as a size marker. The buffer was 1x TAE (50x TAE : 242g Tris, 100mls 0.5M EDTA, pH 8.0, 57.1ml glacial acetic acid / 1 litre), and the gel was run at 150V for approximately 30 minutes. The bands were visualised on a UV transilluminator and photographed.

2.5.4 Analysis PCR Products by Southern Methodology.

2.5.4.1 Southern Blotting.

10µl of the PCR product and 3µl of 10x DNA gel loading dye was run in a 1.5% agarose gel as described above. After visualisation under UV, the gel was denatured for 15-30 minutes in a solution of 0.5M sodium hydroxide and 1.5M sodium chloride, followed by neutralisation in 0.5M Tris and 1.5M sodium chloride for 15-30 minutes. The time spent in the solutions depended on the size of the gel. The PCR products were transferred to Hybond N by placing the gel on 3MM Whatman paper that had been soaked in 10x SSC. The Whatman paper was acting as a wick to a reservoir of 10x SSC. A piece of Hybond N was placed on top of the gel, and any air bubbles removed. The area around the gel was sealed using clingfilm to prevent evaporation of the 10x SSC reservoir. Two pieces of dry 3MM Whatman paper were placed on top of the Hybond N, followed by a pack of paper towels, then a small weight (1kg). Due to the abundance of PCR products in the gel 3-4 hours blotting was all that was

required to transfer enough DNA to the filter that would bind probe during Southern hybridisation. The filter was removed and placed DNA side down on a UV transilluminator for 4 minutes to fix the DNA onto the filter. It was wrapped in clingfilm and stored at 4°C until required.

2.5.4.2 Southern Hybridisation.

The Hybond N filter and a mesh were pre-soaked in 2x SSC, before rolling together and placing in a Hybaid bottle. 10-15mls of PCR prehybridisation buffer (5x SSC, 5x Denhardts', 0.5% SDS, 100μg/μl E.coli tRNA) was added, and the filter incubated at 65°C for 2 hours. The prehybridisation buffer was then replaced by hybridisation buffer (prehybridisation buffer plus 10% dextran sulphate) containing radioactive probe (not less than 10^8cpm/μg), and hybridised for 3-4 hours at 65°C. The probe was made as described in 2.4.4.

2.5.4.3 Southern Washes.

After hybridisation, the filter and mesh were washed in the Hybaid bottle with 2x SSC and 0.1% SDS for 10 minutes at 65°C. They were then removed from the bottle and placed in a box with 0.2% SSC and 0.1% SDS for 15 minutes at 65°C with shaking. If the counts were reduced to between 5 and 20 cps, then the filter was dried slightly, wrapped in clingfilm and placed at -70°C with Kodak X-Omat autoradiographic film and intensifying screens. The film was developed the following day, and if the bands were not intense enough the filter was placed back at -70°C with a new film.

2.6 Generation of cDNA Probes.

2.6.1 Constructs for Generating TGF-β1, TGF-β3, and 7s RNA Probes.

A gene specific mouse TGF-β1 cDNA (Akhurst *et al*, 1990) was used for Northern analysis of TGF-β1 gene expression. A 600bp KpnI - ApaI fragment, corresponding to the region between amino acids 68 - 268 of the LAP, was derived from the full length murine cDNA (Derynck *et al*, 1986). This was subcloned into Bluescribe (Stratagene) in an antisense orientation with respect to the T3 promoter, and isolated using *Pst* I and *Sst* I. The TGF-β3 specific probe was a 738bp PCR fragment, coding for amino acids 8 - 251 of the LAP, subcloned into Bluescript KS II (by P. Kondaiah

& F. Denhez). The T3 promoter generated antisense RNA, while the probe used for Northern analysis was isolated using *Bam* HI and *Eco* RI. The 7s RNA probe was obtained from a cDNA library of mouse embryo small RNAs (Birnie *et al*, 1982). The clone, pA6, contained 190 of the 280 nucleotides represented in 7s RNA, and was isolated using *Hinf* and *Sau* 3a

2.6.2 Transfection of Construct into Bacterial Cells.

20-50ng of plasmid DNA containing the required insert was added to 100µl of DH5α competent cells on ice before heat-shocking at 56°C for 1½ minutes. An incubation on ice for 2 minutes followed before the addition of 0.5ml of autoclaved L-broth (1% bacto-tryptone, 0.5% yeast extract, 1% sodium chloride, pH 7.0), followed by an hour in a 37°C shaker (x 225 rpm). The cells were then plated out at 200, 100, and 50µl volumes onto agar plates (L-broth plus 1.5% bacto-agar and 50mg/ml ampicillin), and incubated at 37°C overnight in an inverted position.

2.6.3 Small Scale Preparation of Plasmid DNA.

During overnight incubation the bacterial cells had proliferated to form colonies on the agar plates. One colony was picked and added to 5mls of pre-warmed L-broth containing 50mg/ml ampicillin. This was incubated with shaking (x 225 rpm) at 37°C overnight. Following the incubation, 1.5mls of culture was placed into an eppendorf and microcentrifuged at 14000 rpm for 5 minutes at room temperature in an Eppendorf benchtop microcentrifuge. The supernatant was removed and the cell pellet resuspended in 100µl GTE (50mM glucose, 25mM Tris HCl, pH 7.4, 10mM EDTA), plus 2mg/ml lysozyme. 200µl of 0.2M sodium hydroxide / 1% SDS was added and left on ice until the solution cleared, approximately 5 minutes. Next, 150µl of KAW (6M potassium acetate, acetic acid, pH5.4) was added, the tubes mixed gently, and then placed on ice for 30 minutes to precipitate the protein. The samples were microcentrifuged for 2-3 minutes at 14000 rpm at room temperature, before removing and retaining the supernatant. An equal volume of isopropanol was added to the solution, and the tubes vortexed, then centrifuged for 5 minutes to precipitate and pellet the DNA. The supernatant was discarded, leaving a pellet, which was then resuspended in 50µl of sterile TE, plus 50µg/ml of RNAseA. This was incubated at 37°C for 30 minutes. Subsequently an equal volume of phenol/chloroform (50:50) was added, the tubes vortexed for 10 seconds to ensure proper mixing, and then centrifuged for 5 minutes to separate out the organic and inorganic phases. The top layer was removed and retained, before adding 1/10th volume of 3M sodium acetate and an equal volume of isopropanol. The tubes were shaken before placing at -20°C

for at least 1 hour to precipitate the DNA again. Microcentrifugation at 14000 rpm followed for 15 minutes to pellet the DNA which was washed 2x in 70% ethanol, air dried, then resuspended in 30μl of TE. 2μl of the sample was run on a 1% agarose gel containing 1μg/ml ethidium bromide to determine the DNA concentration, in conjunction with an optical density measurement using a spectrophotometer. Approximately 0.5-1 μg/μl was obtained from a small scale preparation.

2.6.4 Medium Scale Preparation of Plasmid DNA.

Once the 5ml culture of transfected DH5α bacterial cells was obtained after overnight culture as described in 2.6.2, 200μl was used to inoculate a further 200mls of pre-warmed L-broth plus 50mg/ml ampicillin. After another overnight incubation period at 37°C with agitation (x 225 rpm), the 200ml culture was centrifuged at 3000 rpm for 10 minutes at 4°C in a Sorvall rotor. The supernatant was discarded, and the cell pellet resuspended in 5ml GTE (50mM glucose, 25mM Tris HCl, pH 7.4, 10mM EDTA) before the addition of 40mg/ml lysozyme. After incubation on ice for 15 minutes, 10mls of 0.2M sodium hydroxide / 1% SDS was added, and the solution gently mixed before returning to ice for a further 5 minutes. After the solution cleared, 7.5mls of KAW (6M potassium acetate, acetic acid, pH5.4) was added, the tube inverted, then placed on ice for 20 minutes. A further centrifugation step followed for 10 minutes at 3000 rpm at 4°C in a Sorvall rotor to pellet the protein. This was removed by filtering the solution through a double layer of gauze. The DNA/RNA was then precipitated out of solution by adding 2 volumes of ethanol and standing at room temperature for 5 minutes. The tubes were centrifuged for 10 minutes at 4°C at 3000 rpm to pellet the DNA/RNA. This was then resuspended in 2mls of TE, and transferred to Falcon 2059 centrifuge tubes. 1.6mls of 5M lithium chloride was added, and the tubes placed at -20°C for at least 1 hour. To pellet and remove RNA the samples were centrifuged for 10 minutes at 3000 rpm at 4°C. The supernatant was retained, and 2 volumes of ethanol were added to precipitate the DNA while standing at room temperature for 10 minutes. The DNA was pelleted by centrifugation at 3000 rpm for 10 minutes at 4°C, and was then resuspended in 0.4ml of TE. This solution was removed and placed into a 1.5ml eppendorf tube. An equal volume of phenol was added, and the tubes vigorously vortexed before microcentrifugation for 5 minutes at 14000 rpm at room temperature to separate the layers. The top layer was removed to a fresh 1.5ml eppendorf tube and an equal volume of chloroform was added. Vortexing was required to mix the solutions, before centrifugation at 14000 rpm for 5 minutes to separate out the layers again. The top layer was retained and 2 volumes of ethanol, plus 1/20th volume 4M sodium chloride was added. After shaking to mix the liquids the tubes were put at -20°C for at least an

hour, before spinning for 10 minutes at 14000 rpm at room temperature. The pellet was washed in 80% ethanol, and air dried before the addition of 100µl of TE. The average yield was 2-4µg/µl of DNA.

2.6.5 Restriction Endonuclease Digestion.

To isolate the DNA insert, approximately 20µg of plasmid DNA was combined with the appropriate restriction enzymes, at a concentration of 2-3 units/µg DNA, plus buffer to a total volume of 100µl, and incubated at 37°C. After 1-2 hours 1µg/ml RNAseA was added to remove RNA, and incubation at 37°C continued for a further 30 minutes. 2µl of the digestion products, plus 1/6th volume of gel loading buffer (50% glycerol, 0.25% bromophenol blue, 0.25% xylene cyanol) was run on a 1% agarose gel containing 1.5µg/ml ethidium bromide. 1µg/ml of 1kb DNA ladder was run alongside the samples to confirm the expected size of the insert, and also to estimate its concentration when visualised on a UV transilluminator. If the digestion was correct and complete the total volume of digestion products were run on a 1% low melting point agarose gel for excision and isolation of the insert.

2.6.6 Extraction of DNA Fragments from Low Melting Point Agarose Gels.

To isolate the DNA fragment cut out of the plasmid by restriction endonuclease digestion, the total volume of the digestion was run in a 1% low melting point agarose gel at 50 V for approximately 40-50 minutes. The gel contained 1.5µg/ml ethidium bromide and so allowed visualisation of the products on a UV transilluminator. The band of the correct size was excised from the gel with a sterile scalpel blade and placed into a 1.5ml eppendorf. To remove the DNA from the gel a GeneClean II kit (Bio 101 Inc.) was used. The volume of the gel was measured by weight, and three volumes of 6M sodium iodide added. This was incubated at 50°C for 5 minutes to solubilise the gel, before the addition of 5µl of glassmilk (silica matrix) (5µl per 5µg DNA) and incubation on ice for 5 minutes. The mixture was vortexed every 1-2 minutes to ensure that the glassmilk was exposed to, and binding all of the DNA in solution. The glassmilk/DNA was then pelleted by microcentrifugation at 14000 rpm for 5 seconds at room temperature before washing in 10-50 volumes of NEW wash (sodium chloride, Tris, EDTA, ethanol mixture in kit). The tube was microcentrifuged for 5 seconds at 14000 rpm to pellet the glassmilk/DNA again, and the supernatant removed. The washing step was repeated twice more before the pellet was resuspended in an equal volume of distilled water and incubated at 50°C for 2-3 minutes to release the DNA from the glassmilk. The glassmilk was pelleted, and the

water containing the DNA was removed to a fresh eppendorf. The elution step was performed twice. 1μl of the sample was run in a 1% agarose gel to check that the DNA was still present, and to estimate the concentration.

Chapter 3

RESULTS

3.1 Analysis of TGF-β Levels in Serum and Plasma using the CCL64 Assay.

The aim of this project was to investigate the function of TGF-β, specifically TGF-β1, during embryonic development by depleting the embryo of TGF-β1 using either antisense technology or transgenic knockout technology. *In vitro* post-implantation embryo culture was to be utilised to examine the effect of the removal of endogenous TGF-β1 protein on embryonic development. Since any exogenous TGF-β protein may have had an effect on the TGF-β1 depleted embryos, a medium which lacked TGF-β had to be found. Several different combinations of media were tried, and the biological fluids, serum and plasma, were analysed for TGF-β content using the CCL64 assay. This bioassay for TGF-β was devised in 1989 by Danielpour *et al* based on the fact that mink lung epithelial cells are potently growth inhibited by TGF-β. The cell proliferation rate, as measured by tritiated thymidine incorporation, therefore reflects the amount of TGF-β present in the sample. In this project, known quantities of recombinant TGF-β1 were used as standards, and the serum and plasma samples were measured at several dilutions; 1, 10, 20, and 100μl in a total volume of 200μl of assay buffer. Each sample and standard was assayed in triplicate in each experiment. Four different assays on 3 different serum preparations were performed to determine the concentration of TGF-β1 in rat serum. Likewise, 6 independent assays on 5 different plasma preparations were used to determine the concentration of TGF-β in rat plasma.

3.1.1 CCL64 Assay to Measure TGF-β1 in Rat Serum.

Before the CCL64 assay was performed, the serum was extracted from rat blood, as described in Materials and Methods, and heated for a further 15 minutes at 94°C. This step was required to heat-activate any latent TGF-β, and also inactivate any heat-sensitive growth stimulatory factors, such as TGF-α. These stimulatory factors were previously discovered in a CCL64 assay comparing heated versus un-heated serum (Fig.5). Fig.6 is representative of all the assays performed and shows the results of one CCL64 assay, analysing 1, 10 and 20μl dilutions of rat serum in 200μl of assay buffer. Inhibition of cell proliferation was observed when the rat serum samples were added, indicating that TGF-β was present. However, this inhibition was attenuated with increasing amounts of rat serum. This probably indicated the presence of heat-stable growth stimulatory factors which were able to override the negative effects of serum TGF-β. Thus estimates of TGF-β concentration were only minimal estimates.

The data from the 1μl samples were used to estimate serum TGF-β1 concentration since these contain the lowest levels of interfering growth stimulatory factors. Furthermore, to assist in the evaluation of the fraction of growth inhibitory activity attributable to TGF-β1, TGF-β1 blocking antibody was utilised. The amount of TGF-β1 was measured as the difference between the samples in the presence or absence of 10mg/ml TGF-β1 antibody. The antibody alone had no stimulatory or inhibitory effect on the cells. When 1μl of rat serum was analysed and compared to the standard curve, the result suggested that there was 125pg/ml of TGF-β1 in the sample. When 1μl rat serum plus antibody was analysed, there was an increase in cell proliferation, and comparison with the standard curve suggested that there was 50pg/ml of TGF-β1 (Fig.6). It appeared therefore, that the antibody had bound to and removed the equivalent of approximately 75pg/ml TGF-β1. When the dilution factor was taken into consideration, the results of the assays suggested that there was at least 15ng/ml TGF-β1 in rat serum. This was excessive in quantity compared to an ED_{50} of 50-75pg/ml for many biological activities. Serum could therefore not be used in a medium for culturing TGF-$\beta^{-/-}$ embryos.

3.1.2 CCL64 Assay to Measure TGF-β1 in Rat Plasma.

TGF-β1 levels in rat plasma were analysed using the CCL64 assay. The plasma was extracted from blood as described in Materials and Methods, and centrifuged twice at 14000 rpm for 15 minutes to remove any platelets. This doubly centrifuged plasma (dCP) was assayed for TGF-β1 concentration after heating at 94°C for 15 minutes to heat-activate any latent TGF-β, and inactivate any heat-sensitive growth stimulatory

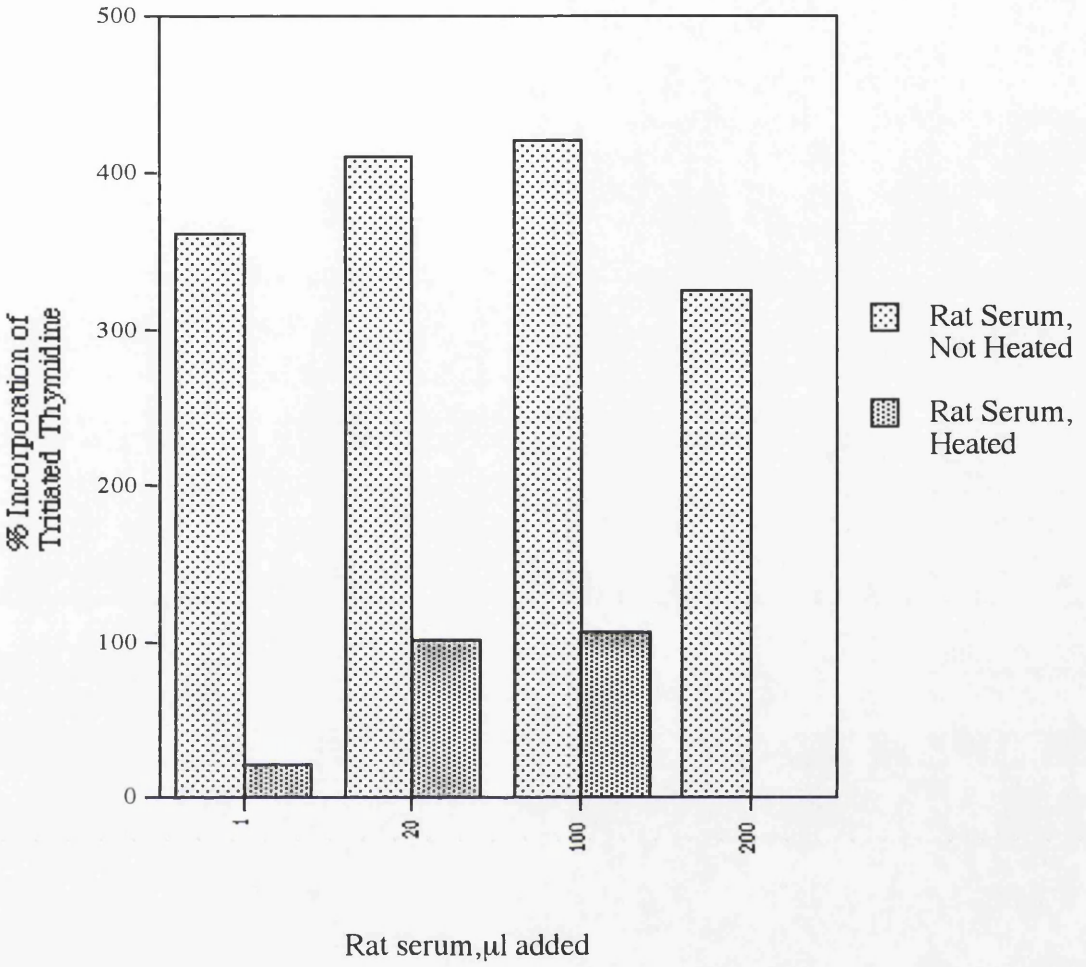


Figure 5 - Growth Stimulatory Effect of Rat Serum.

Rat serum was analysed by CCL64 assay to determine the presence of any heat sensitive stimulatory factors. 1, 20, and 100μl samples, in a total of 200μl with assay buffer, were either heated at 94°C for 15 minutes, before quenching on ice, or were left at room temperature. They were then assayed in the CCL64 assay, and the level of cell stimulation measured by tritiated thymidine incorporation. 100% tritiated thymidine incorporation represents the level with no added components.

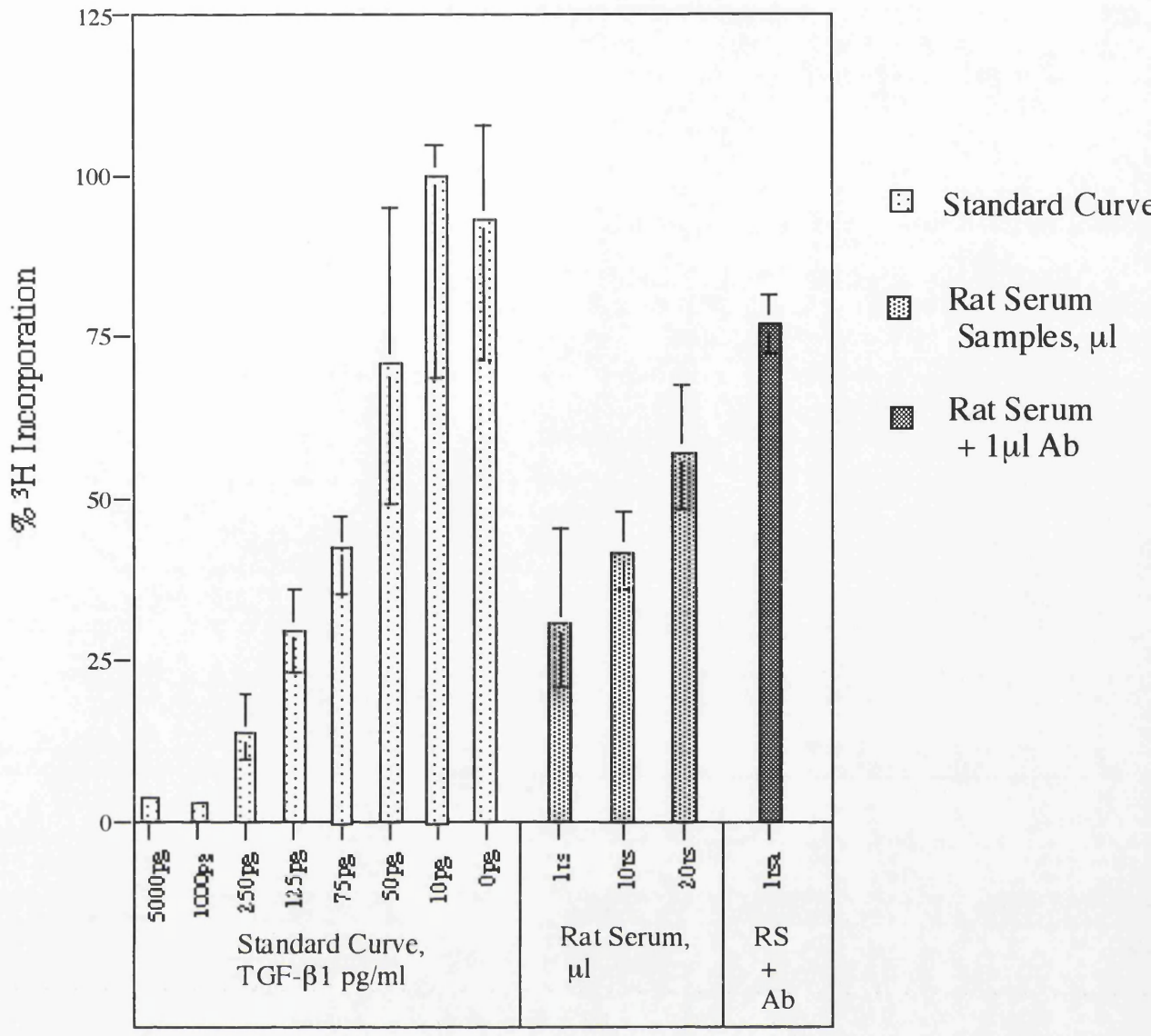


Figure 6 - Concentration of TGF-β in Rat Serum.

The CCL64 assay was used to determine the amount of TGF-β in rat serum (Danielpour *et al*, 1989). Known concentrations (5000pg-0pg/ml) of recombinant TGF-β1 (rTGF-β1) were assayed to generate a standard "curve". 1, 10, or 20µl of rat serum, in a total volume of 200µl with assay buffer, were analysed in the same assay. Each standard concentration and rat serum sample was performed in triplicate. To determine the approximate amount of TGF-β1 in the rat serum, 10mg/ml anti-TGF-β1 antibody was added to 1µl rat serum. On comparison with the standard curve, there appears to be approximately 125pg/ml TGF-β1 in 1µl of rat serum. With antibody, the stimulation was comparable with 50pg/ml rTGF-β1. This therefore suggests that the antibody had bound to and removed 75pg/ml TGF-β1. Taking the dilution factor into consideration, there is therefore a minimum of 15ng/ml TGF-β1 present in rat serum.

RS - rat serum; Ab - anti-TGF-β1 antibody.

factors. Fig.7 is representative of all six assays performed for dCP and shows the results of one assay using 1, 10, and 20μl dilutions. Tritiated thymidine incorporation was consistently higher in the cells exposed to the plasma samples in comparison to the cells exposed to 10 or 0pg/ml of rTGF-β1 (Fig.7), suggesting that there was no TGF-β in the plasma samples. To test the possiblity that there was a very small amount of TGF-β1 present, but that its inhibitory effect was masked by stimulatory factors, 10mg/ml TGF-β1 antibody was added to the samples (Fig.7). An increase in tritiated thymidine incorporation by the cells was not observed, further supporting the finding that there was no detectable TGF-β1 in the plasma. dCP therefore appeared suitable for use as a medium which lacked TGF-β1 (ie.< 10pg/ml).

As a potential candidate for culturing post-implantation embryos, plasma appeared ideal. Although it was not fully defined, it was a biological fluid and so contained many essential components such as glucose, vitamins, and other growth factors that are essential for the development of younger embryos *ex utero*. However, for this project, the most important factor was that dCP contained undetectable levels of TGF-β.

There was one major problem though. Other investigators had previously found plasma unsuitable for culturing embryos (Kesby, 1992). Kesby found that with an increase in the amount of soluble heparin used to prevent the blood from clotting during the extraction procedure, there was a concomitant rise in the number and severity of embryonic abnormalities, with significant defects first observed at 20 IU/ml. However, in this study the heparin was not added as a liquid to the blood, but rather as heparin-coated balls, and at a concentration of 10 IU/ml. Whether or not plasma prepared in this way would also be toxic to embryos could only be discovered by *in vitro* culture.

3.2 Characterisation of a Post-Implantation Embryo Culture System.

Before experiments investigating the role of TGF-β in development could be performed, the ability of dCP to support embryonic development had to be assessed. Due to the undefined nature of the plasma, it seemed sensible to try and reduce the quantity used in the culture medium, thereby decreasing the amount of unknown factors. This would also decrease the amount of heparin that the embryos would be exposed to. Therefore, instead of using 100% dCP, several different quantities of dCP were tried and tested in combination with one of two chemically-defined, commercial

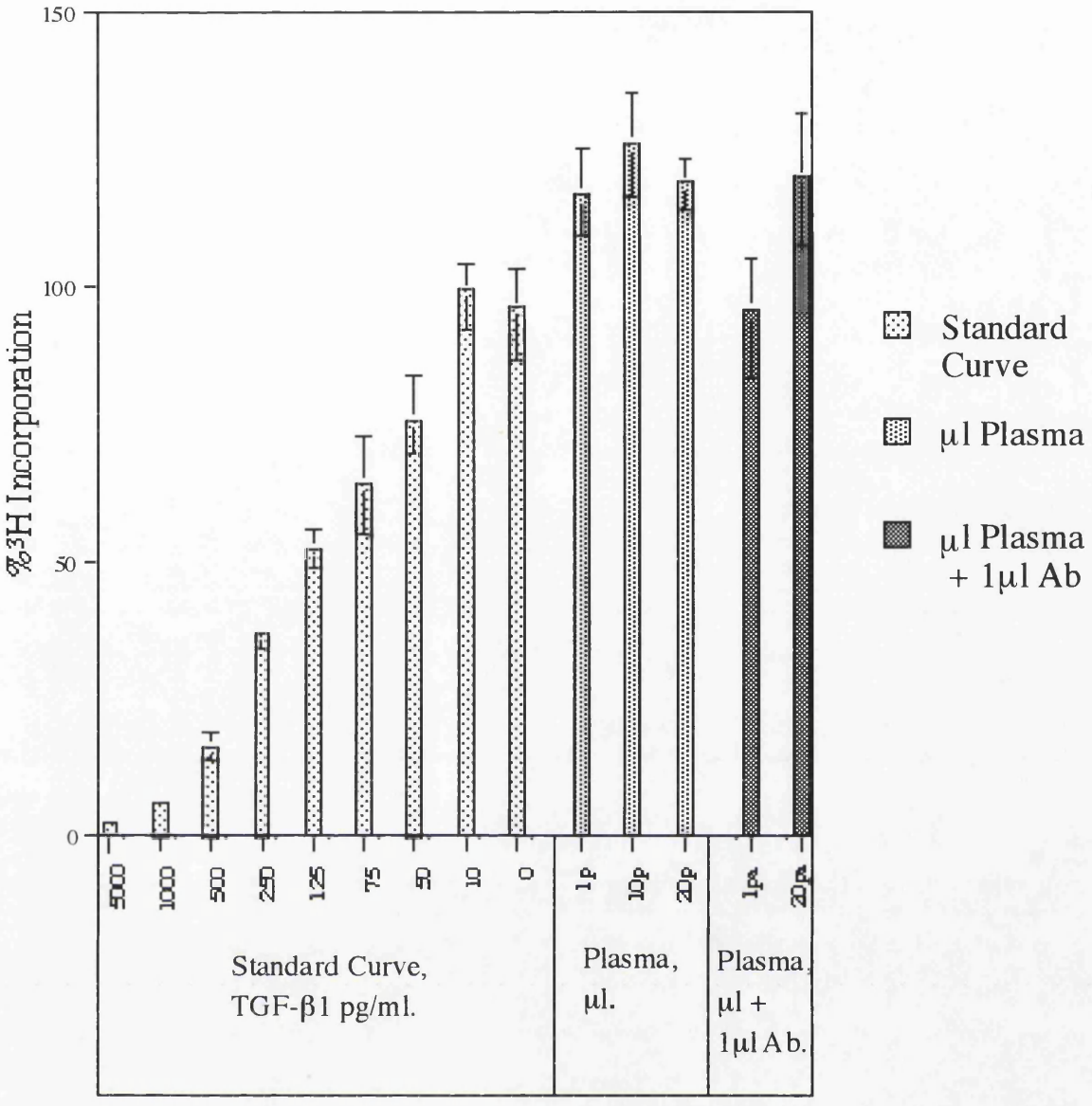


Figure 7 - TGF-β Concentration in Rat Plasma.

The CCL64 assay was used to determine the amount of TGF-β1 in rat plasma (Danielpour *et al*, 1989). Known concentrations (5000pg-0pg/ml) of recombinant TGF-β1 were assayed to generate a standard "curve". 1, 10, 20µl rat plasma samples in a total volume of 200µl with assay buffer, were analysed in the same assay. Each TGF-β standard and rat plasma sample was assayed in triplicate. To determine the approximate amount of TGF-β1 in the rat plasma, 10mg/ml anti-TGF-β1 antibody was added to 1µl plasma samples. As shown above, the results indicated that there was no TGF-β1 present in the plasma samples. The cells were not inhibited in the presence of the plasma, and there was no increase in stimulation when the antibody was added.

media; Dulbecco's Modified Eagles Medium (DMEM) or Foetal Mouse Palate Medium (FMP) (Table 1, 2). As the name suggests, FMP was originally used to culture and study the development of mouse embryo palates (Shiota *et al*, 1990), and so may have contained components suitable for supporting whole embryo development. Rat serum was also used in combination with these media to compare with the dCP samples.

NIH or Parkes/NIH mice were used in the initial studies to test the different culture media. After culture, the embryos were analysed using the Brown-Fabro scoring system (Brown & Fabro, 1981) (Table 3), as described in Materials and Methods. However, although several critical embryonic characterisics are assessed using this system, the resulting final score is only a reflection of the developmental stage of the embryo, and does not reveal whether the embryo has developed normally. For example, an embryo may have the physical attributes of a 9.5 dpc embryo, but one of these attributes may be abnormal. Therefore, in this project, abnormalities were also assessed, and subdivided into "major" and "minor" abnormalities. "Major" abnormalities were defects which would seriously affect embryonic development, for example, the persistence of open headfolds, whereas "minor" abnormalities were small, non-offensive defects, such as a tiny spot of blood in the forebrain, which would not affect the continued short-term development of the embryo.

3.2 1 The Use of Standard Embryos as Controls.

A panel of embryo standards was generated using uncultured embryos, against which the cultured embryos could be compared. 194 Parkes/NIH or NIH embryos were removed from the uterus at a variety of ages (8, 8.25, 8.5, 8.75, 9, 9.25, 9.5, 9.75 and 10 dpc) (Fig.8), and scored by the Brown-Fabro method. Variation between the embryos of a certain age group was observed, as indicated by the standard error bars in Fig.8, as well as variation between embryos of the same litter. This latter observation is a common phenomenon as not all embryos develop at the same rate *in utero*. However, the difference was at most 1/4 of a day.

3.2.2 7.5 dpc Embryo Culture.

The different media were initially analysed for their ability to support normal embryonic development from 7.5 to 8.5 dpc *in vitro*. 271 NIH or NIH/Parkes embryos were cultured in the media for 24 hours, scored and then assessed for abnormalities.

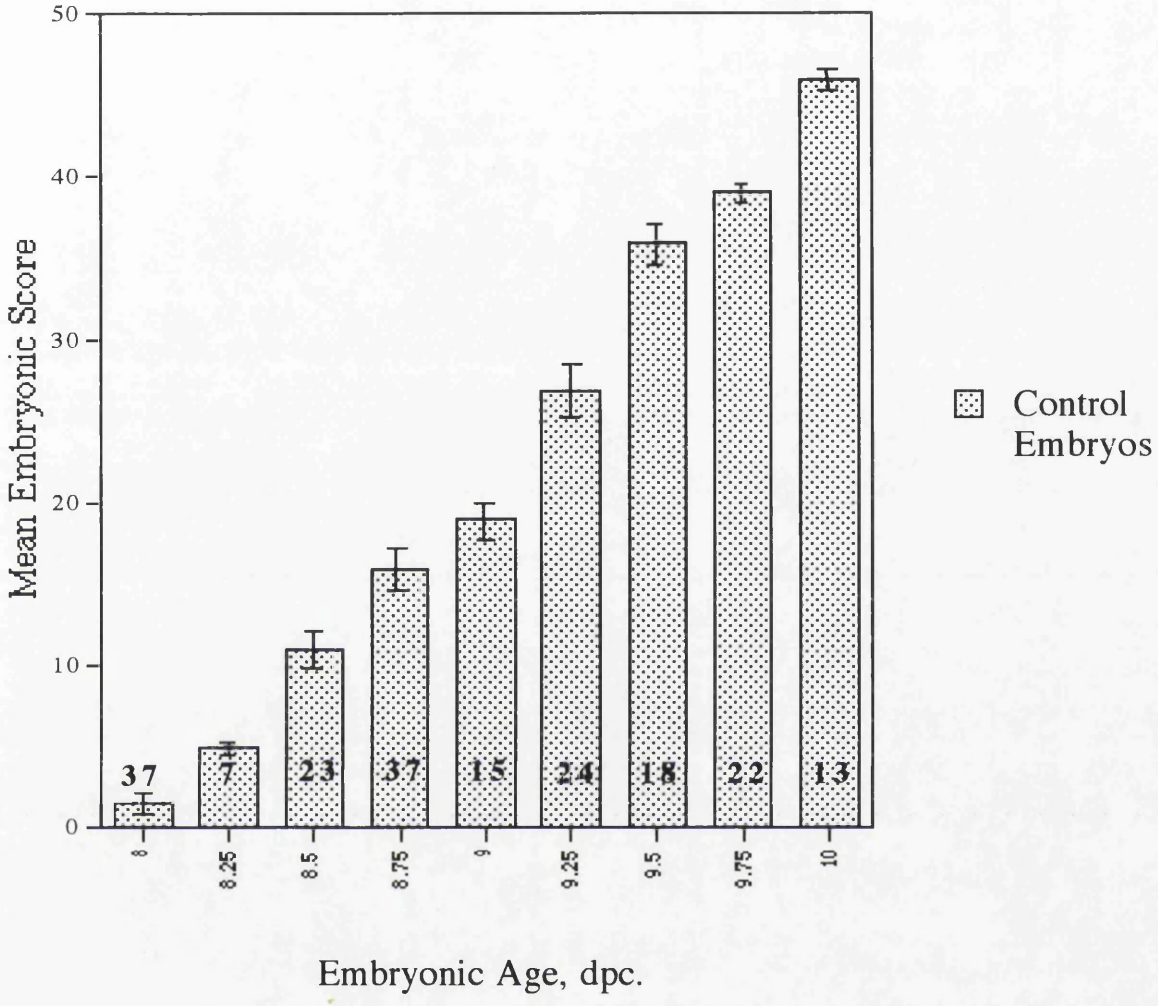


Figure 8 - Morphological Analysis of Control Embryos.

Uncultured embryos, ranging from 8.0-10.0 dpc were removed from the uterus, and assessed using the Brown-Fabro scoring method (1981). A number of embryonic characterisitics are each given a score depending on their appearance. These values are then added together to give the embryos score. A higher score indicates a more advanced embryo. Each column in the graph represents the mean score for the embryos of that age.The number in each column represents the number of embryos examined. Vertical bars show the standard error of the mean.

DMEM : 50% rat serum is commonly used by other groups (Tam & Beddington, 1987; Savatier *et al*, 1990; Shepard *et al*, 1993), and supported the development of 7.5 dpc embryos very efficiently in this study (Fig.9, 10). The rate of development was slightly delayed by approximately 2-3 hours, and there were a few necrotic embryos, but these abberations were not significant when compared with controls. From 30 embryos cultured in DMEM : 50% rat serum, 6 were slightly necrotic. If the amount of rat serum was decreased to 10%, the embryos continued to grow over the 24 hour time period, but both the delay in development, and the number of abnormalities increased very slightly (Fig.9, 10, Table 5). If DMEM was combined with dCP, embryonic development was considerably delayed, at most attaining the stage of 8.25 dpc (Fig.9). This was observed for the majority of the 25 embryos cultured in DMEM : 50% dCP, and the 26 embryos cultured in DMEM : 10% dCP. The most dramatic effect however, was the observed increase in abnormalities when DMEM : 50% dCP was used (Fig.11, Table 5). Major abnormalities were observed in 6/30 and 5/30 embryos cultured in DMEM : 50% rat serum and DMEM : 10% rat serum, respectively, whereas, when DMEM : 50% dCP was used, 16/25 embryos were severely abnormal. Table 5 shows the range of abnormalities observed.

Although necrosis was probably a secondary effect, it was the most commonly observed problem, accounting for 10 out of the 16 abnormal embryos resulting from culture in DMEM : 50% dCP. Although necrotic embryos were observed after culture in the other media, it was most severe in the embryos from DMEM : 50% dCP. The cells necrosed until embryonic structures were unidentifiable, and only a yellow, sticky, ball of cells remained. Other abnormalities included splayed head folds, which commonly appeared as if on stalks, and twisted somites or even a total lack of somites. When the dCP was decreased to 10%, the range of defects were still observed but the number of abnormal embryos decreased to 8 out of 26.

Embryos cultured in FMP : 50% rat serum, and FMP : 10% rat serum developed to between 8.25 and 8.5 dpc and exhibited very few abnormalities, 4/26 and 10/67 respectively (Fig.9, 10, 11, Table 5). When FMP : 50% dCP was used, the embryos developed at the normal rate for the 24 hours *in vitro*, attaining the characteristics of 8.5 dpc embryos, but 8 of the 32 embryos had some sort of serious defect. The range of defects observed was as for the DMEM : 50% dCP cultured embryos, but the majority had headfold and somite phenotypes, rather then necrosis (Table 5). When FMP : 10% dCP was used, 18/31 embryos had a major abnormality, 17 of which were necrotic.

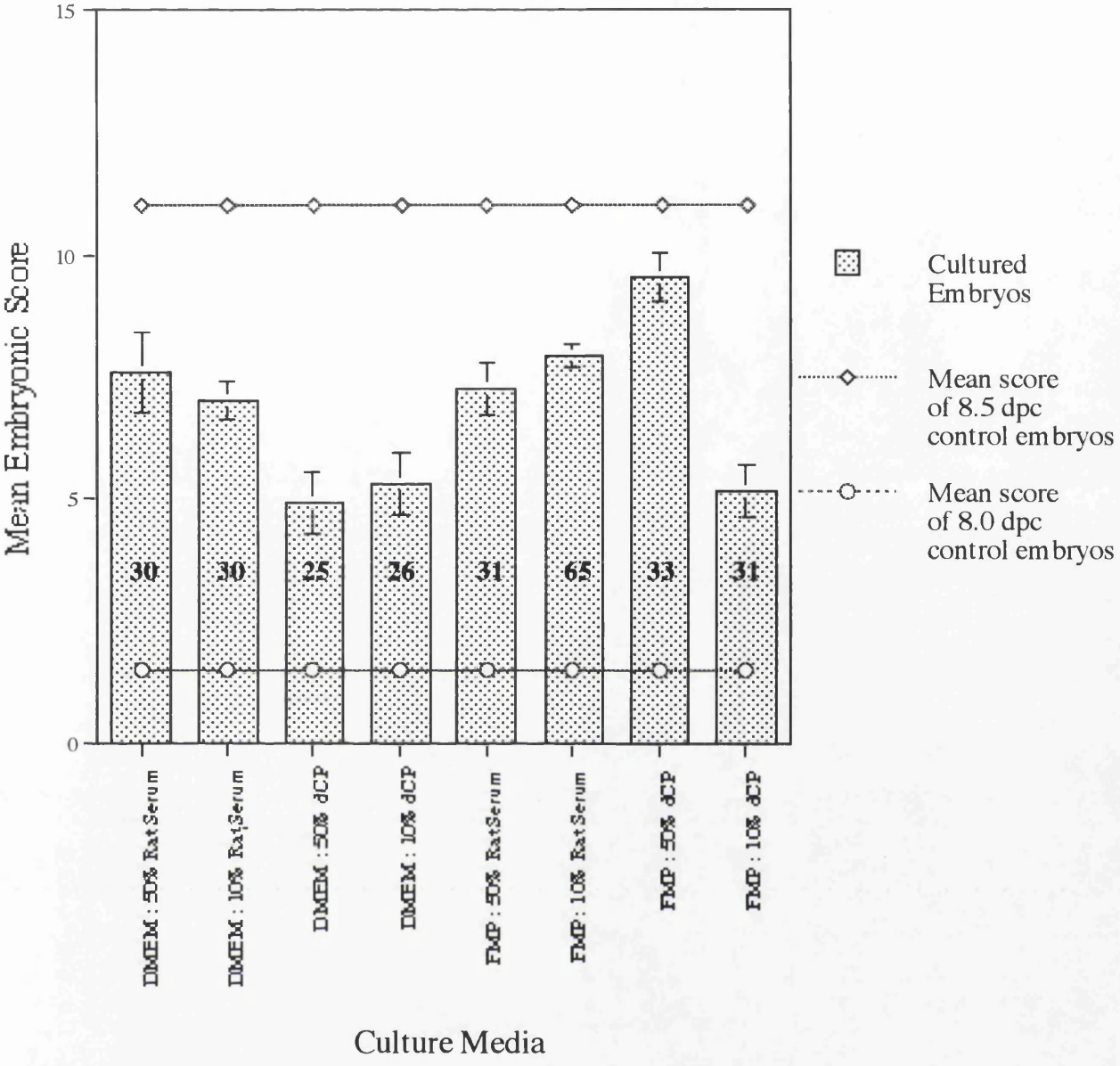


Figure 9 - 7.5 dpc Embryo Culture.

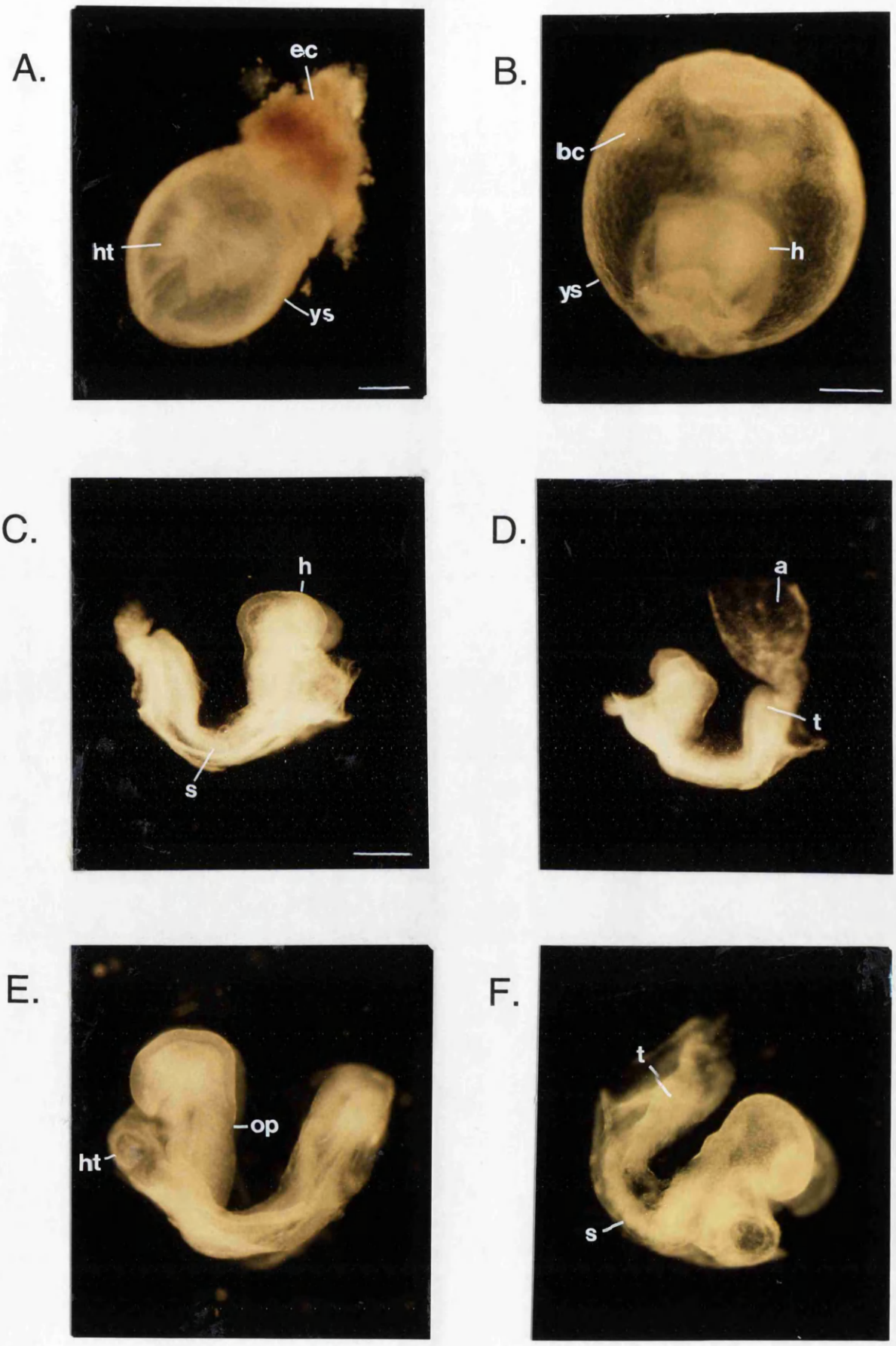
7.5 dpc embryos were removed from the uterus and cultured in a variety of media for 24 hours. After culture, the development of the embryos was assessed by the Brown-Fabro method (1981). The graph shows the mean score for embryos cultured in each of the media. The number in each columns represents the number of embryos cultured in that medium, and the vertical bars are the standard error of the mean. The mean score for 8.0 and 8.5 dpc control embryos are also shown.

Figure 10 - Morphological Analysis of Cultured 7.5 dpc Embryos.

Embryos were removed from the uterus at 7.5 dpc and cultured in a one of a variety of media for 24 hours. (A) 8.5 dpc control and (B) an embryo after culture in FMP : 50% rat serum. Note the developing corona in the yolk sac, spreading down from the ectoplacental cone region. This was also present in the yolk sacs of embryos (C) - (F). The embryo in (C) was cultured in FMP : 50% dCP, and is slightly older than the others. (D) represents embryos after culture in either FMP : 10% rat serum , or 10% dCP, while (E) is representative of culture in DMEM : 50% rat serum The embryo in (F) was cultured in FMP : 10% rat serum, and is similar to those cultured in DMEM : 10% dCP.

a - allantois; bc - blood corona; ec - ectoplacental cone; h - headfolds; ht - heart tube; op - otic pit; s - somites; t - tail; ys - yolk sac.

Scale bars : (A,E,F) - 320μm ; (B) - 280μm ; (C,D) - 300μm.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	Total Number of Embryos Cultured.	Number of Embryos that Developed Well.	Number of Embryos with Major Defects.	Number of Embryos with Minor Defects.	Number of Embryos with Major Headfold Defects.	Number of Embryos with Major Somite Defects.	Number of Necrotic Embryos.
DMEM : 50% Rat Serum	30 (100%)	24 (80%)	6 (20%)	0 (0%)	0 (0%)	0 (0%)	6 (20%)
DMEM : 10% Rat Serum	30 (100%)	23 (77%)	5 (17%)	2 (6%)	2 (6%)	1 (3%)	2 (6%)
DMEM : 50% dCP	25 (100%)	3 (12%)	16 (64%)	6 (24%)	2 (8%)	5 (20%)	10 (40%)
DMEM : 10% dCP	26 (100%)	15 (58%)	8 (31%)	3 (12%)	0 (0%)	0 (0%)	8 (31%)
FMP : 50% Rat Serum	26 (100%)	22 (85%)	4 (15%)	0 (0%)	0 (0%)	0 (0%)	4 (15%)
FMP : 10% Rat Serum	67 (100%)	57 (85%)	10 (15%)	0 (0%)	1 (1.5%)	0 (0%)	9 (13%)
FMP : 50% dCP	32 (100%)	21 (66%)	8 (25%)	3 (9%)	1 (3%)	6 (19%)	1 (3%)
FMP : 10% dCP	31 (100%)	12 (39%)	18 (58%)	1 (3%)	5 (16%)	2 (6%)	17 (55%)

Table 5 - Abnormalities Observed After 7.5 dpc Embryo Culture.

This table shows the number of 7.5 dpc embryos cultured in each medium, the number which developed abnormalities, and whether these abnormalities were major or minor. The major abnormalities observed in these embryos after culture were headfold defects, such as anencephaly; somite defects, such as missing somites; or necrosis. The number of embryos with each of these defects in each medium is also noted.

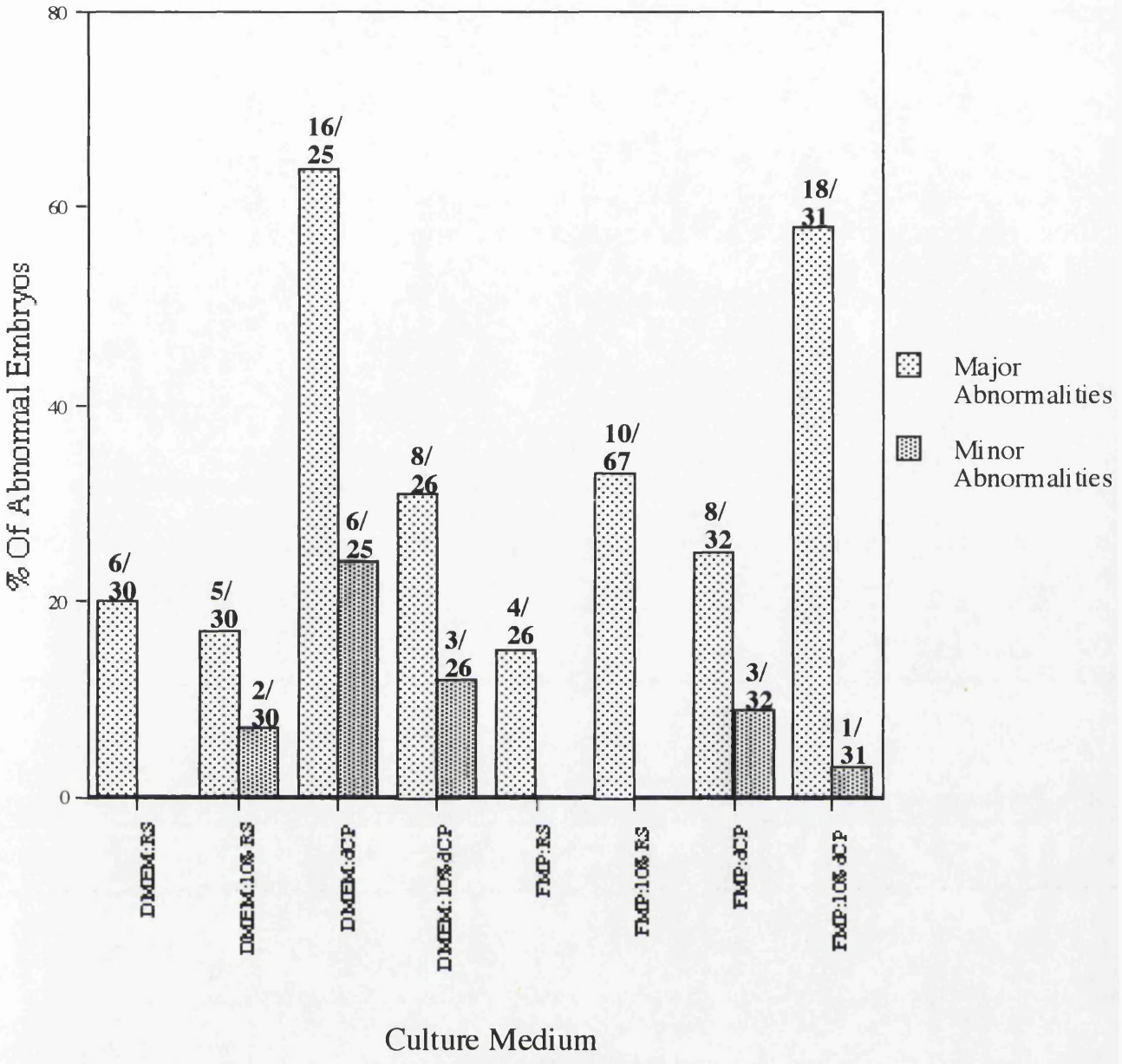

Figure 11 - Abnormalities Observed in Cultured 7.5 dpc Embryos.

7.5 dpc embryos were cultured for 24 hours before their *in vitro* development was assessed by scoring and analysis of abnormalities. A number of abnormal phenotypes were observed, and classified into two groups; major, or minor. Major abnormalities were defects that would have resulted in death relatively quickly had the culture continued, whereas minor abnormalities would not have. The graph above shows the number of embryos with major or minor abnormalities after culture in each medium. The figures above each column represent the number of abnormal embryos / the total number of embryos cultured.

To determine whether these developmental differences were significant, the scores of the embryos cultured in each media were analysed and compared by a students t-test. (Appendix A1). In brief, there was a significant difference ($P \leq 0.05$) between the embryos grown in several of the media, especially between the media containing either DMEM or FMP, and between DMEM plus serum, and DMEM plus dCP.

The results of the 7.5 dpc embryo cultures suggested that DMEM : 50% rat serum and FMP : 50% rat serum were the best media in which to culture embryos of this age. They were equally effective at supporting development, maintaining normal embryonic growth to between 8.25 and 8.5 dpc. However, both of these contain serum, and therefore, TGF-b. FMP : 50% dCP did not contain TGF-b, and although the embryos developed at the same rate as their *in vivo* counterparts, there was a higher incidence of abnormality. It was therefore difficult to obtain normal *in vitro* development of 7.5 dpc embryos in a medium lacking TGF-β.

3.2.3 8.5 dpc Embryo Culture.

Parkes/NIH and NIH embryos were removed from the uterus at 8.5 dpc and cultured in a variety of media (Table 2) to determine which, if any, provided the best support for *in vitro* embryonic development over the 24 hours from 8.5 dpc to 9.5 dpc. 261 embryos were used in this study.

After 24 hours, the majority of embryos cultured in DMEM : 50% rat serum, and DMEM : 10% rat serum had developed to approximately 9.25 dpc (Fig.12, 13). However, 6 of the 23 embryos cultured in DMEM : 50% rat serum, and 5 of the 23 embryos cultured in DMEM : 10% rat serum exhibited some major abnormality (Fig.13, 14, Table 6). 5/6 abnormal embryos cultured in DMEM : 50% rat serum, and 5/5 in DMEM : 10% rat serum had died during the culture period and were necrotic. These necrotic embryos had a grainy appearance, which did not compare to the severity of necrosis that was observed in the 7.5 dpc embryos. Some embryos also had abnormal forebrain regions, such as open or twisted brainfolds (Table 6). A grainy or bumpy yolk sac was found in 1 of the embryos cultured in DMEM : 50% rat serum, and in 5 of the DMEM : 10% rat serum embryos. When DMEM was combined with dCP embryonic development was delayed and even failed to reach 9.25 dpc (Fig.12, 13). 10 out of 23 embryos exhibited serious abnormalities; 7 were necrotic, and 4 exhibited abnormal forebrain phenotypes (Fig.13, 14, Table 6). These forebrain phenotypes included anencephaly (open brain folds) or hydrocephaly (ballooned forebrain cavities). When the amount of dCP was reduced to 10%, the *in vitro* development of the embryos improved (Fig.12). They developed to between

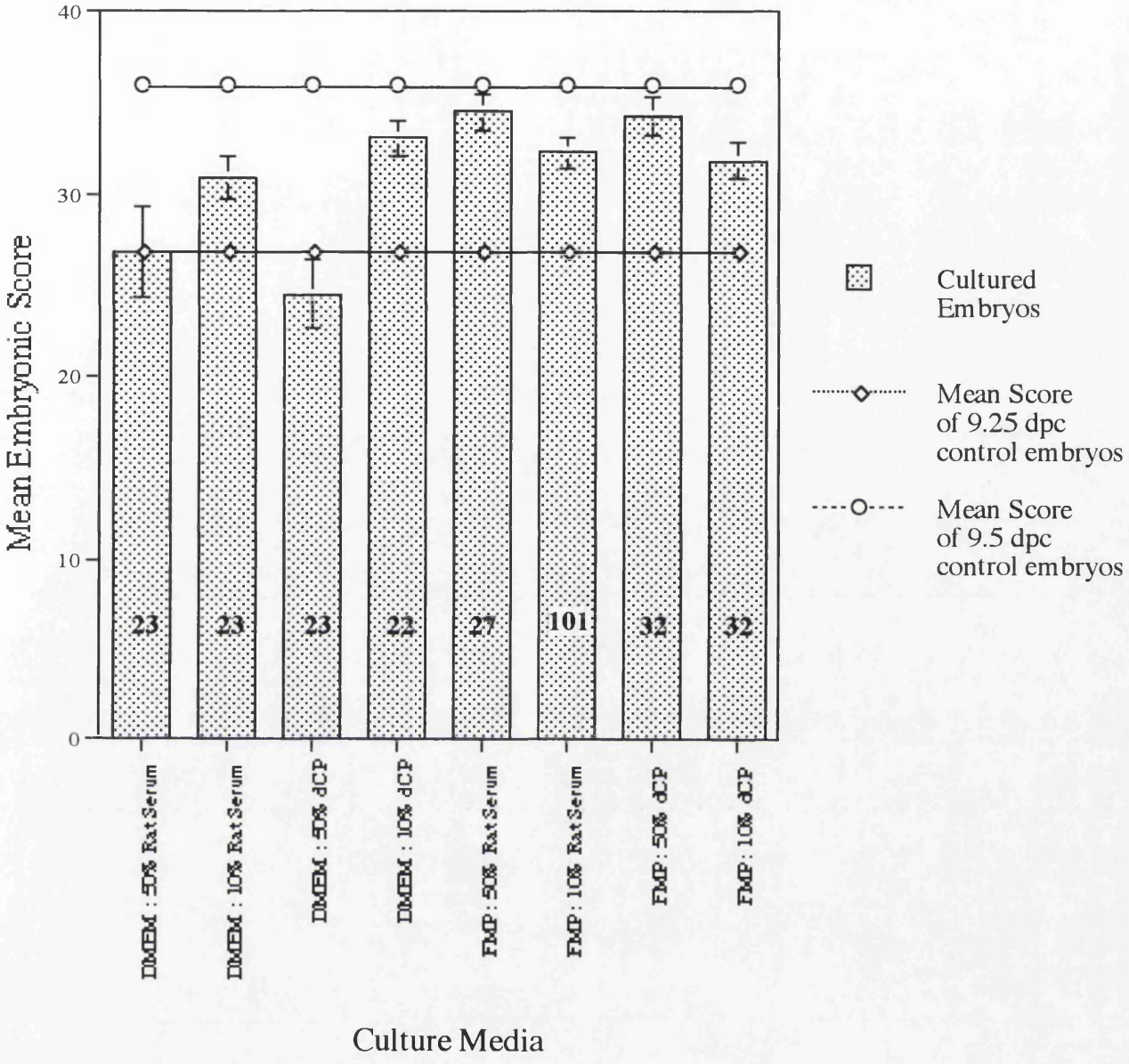

Figure 12 - 8.5 dpc Embryo Cultures.

8.5 dpc embryos were removed from the uterus and cultured in a variety of media for 24 hours. After culture, the development of the embryos was assessed by the Brown-Fabro method (1981). The graph shows the mean score for embryos cultured in each medium. The number of embryos cultured in each medium is represented by the number in each column, and the vertical bars are the standard error of the mean. The mean score for 9.25 and 9.5 dpc control embryos are also shown..

Figure 13 - Morphological Analysis of Cultured 8.5 dpc Embryos.

Embryos were removed from the uterus at 8.5 dpc and cultured in a one of a variety of media for 24 hours. (A) 9.5-9.75 dpc control and (B) an embryo after culture in FMP : 50% rat serum. The embryo in (C) was cultured in FMP : 50% dCP, and (D) represents embryos after culture in either FMP : 10% rat serum, or 10% dCP. (E) Embryo cultured in DMEM : 50% rat serum, and (F) embryo cultured in DMEM : 50% dCP.

b - blood; bb - branchial bars; da - dorsal aorta; h - heart; lb - limb bud; oh - open headfolds; otv - otic vesicle; ov - optic vesicle; pn - posterior neuropore; s - somites.

Scale bars : (A) - 500μm ; (B-F) - 625μm.

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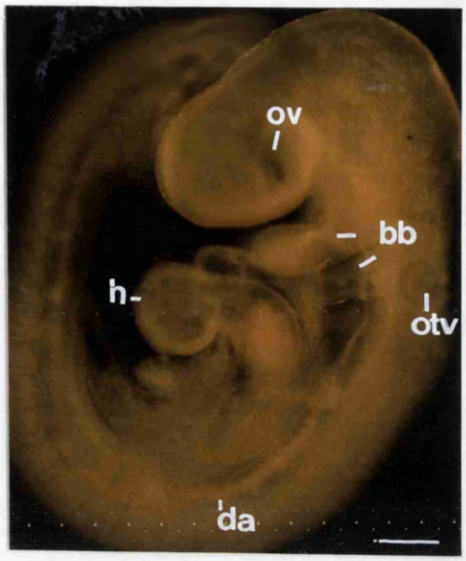

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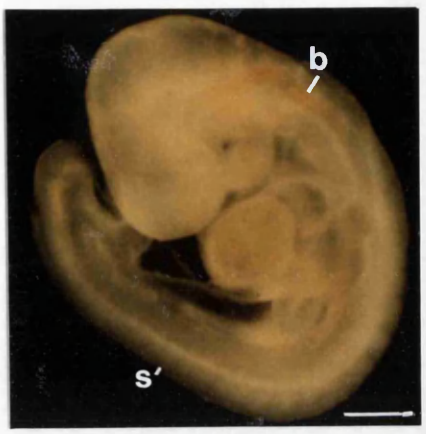

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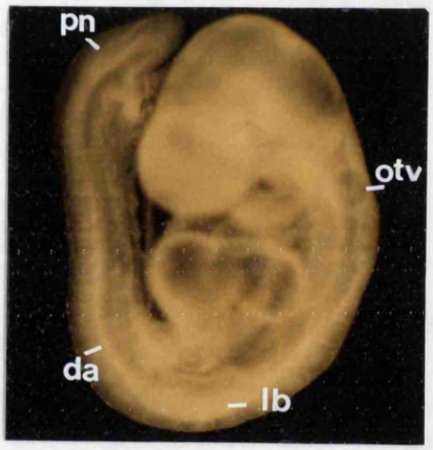

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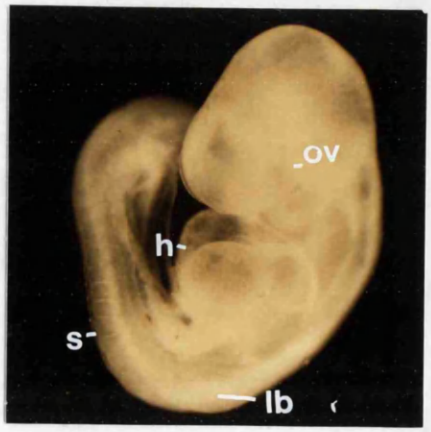

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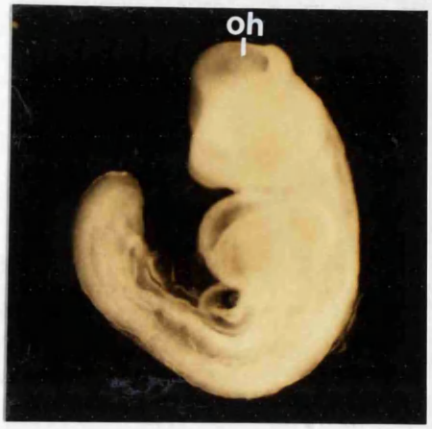


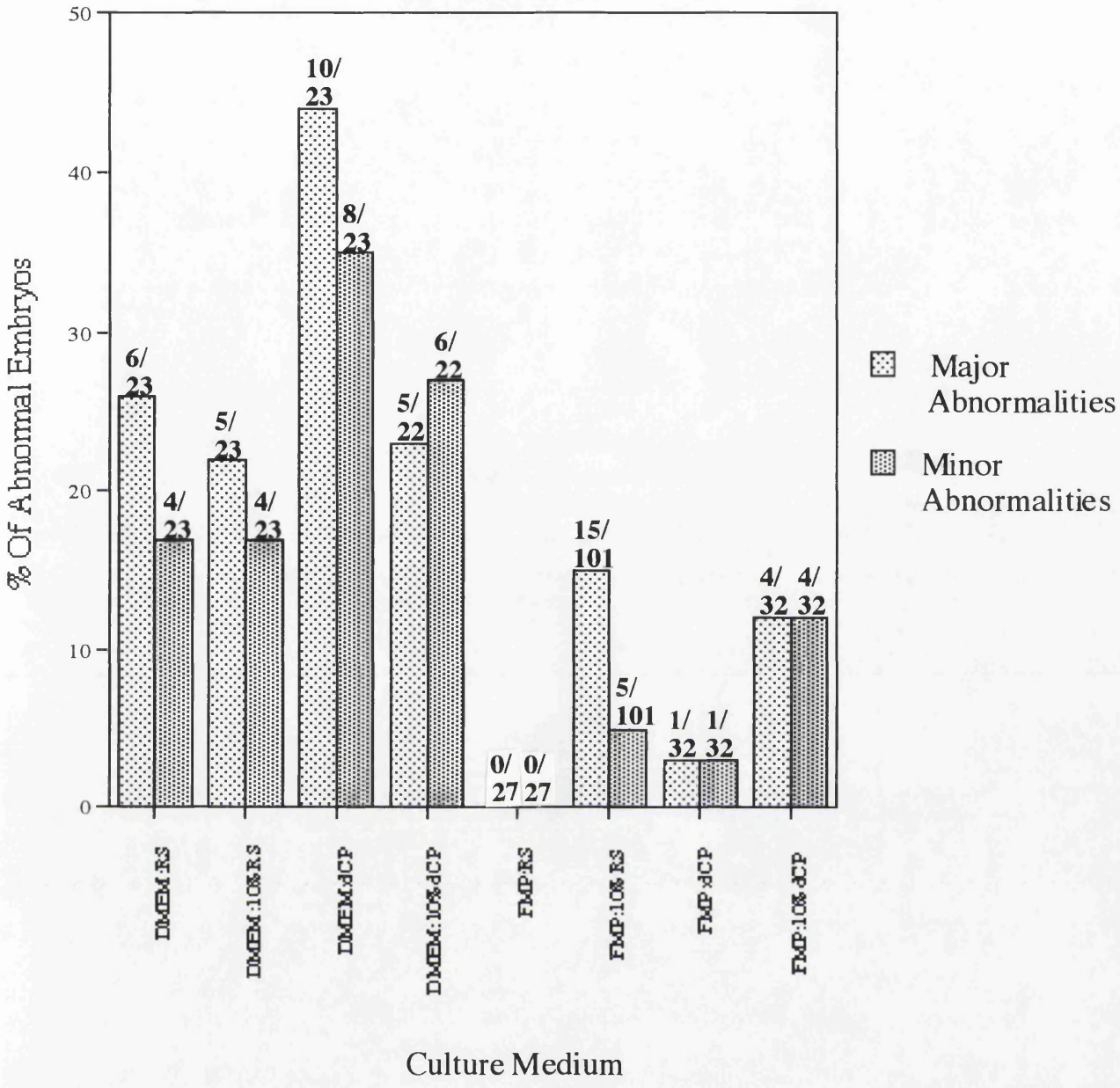


Figure 14 - Abnormalities Observed in Cultured 8.5 dpc Embryos.

8.5 dpc embryos were cultured for 24 hours before their *in vitro* development was assessed by scoring and analysis of abnormalities. A number of abnormal phenotypes were observed, and classified into two groups; major, or minor. Major abnormalities were defects that would have resulted in death relatively quickly had the culture continued, whereas minor abnormalities would not have. The graph above shows the number of embryos with major or minor abnormalities after culture in each medium. The figures above each column represent the number of abnormal embryos / the total number of embryos cultured.

	Total Number of Embryos Cultured.	Number of Embryos that Developed Normally.	Number of Embryos with Major Defects.	Number of Embryos with Minor Defects.	Number of Embryos with Major Headfold Defects.	Number of Embryos with Major Heart Defects.	Number of Embryos with Major Yolk Sac Defects.	Number of Necrotic Embryos.
DMEM : 50% Rat Serum	23 (100%)	13 (57%)	6 (26%)	4 (17%)	3 (13%)	0 (0%)	1 (4%)	5 (22%)
DMEM : 10% Rat Serum	23 (100%)	14 (61%)	5 (22%)	4 (17%)	2 (9%)	1 (4%)	5 (22%)	5 (22%)
DMEM : 50% dCP	23 (100%)	5 (22%)	10 (44%)	8 (35%)	4 (17%)	1 (4%)	4 (17%)	7 (30%)
DMEM : 10% dCP	22 (100%)	11 (50%)	5 (23%)	6 (27%)	3 (14%)	1 (4%)	0 (0%)	5 (23%)
FMP : 50% Rat Serum	27 (100%)	27 (100%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
FMP : 10% Rat Serum	101 (100%)	81 (80%)	15 (15%)	5 (5%)	0 (0%)	0 (0%)	1 (0.01%)	15 (15%)
FMP : 50% dCP	32 (100%)	30 (94%)	1 (3%)	1 (3%)	0 (0%)	0 (0%)	0 (0%)	1 (3%)
FMP : 10% dCP	32 (100%)	24 (75%)	4 (12.5%)	4 (12.5%)	0 (0%)	0 (0%)	0 (0%)	4 (12.5%)

Table 6 - Abnormalities Observed after 8.5 dpc Embryo Culture.

This table shows the number of 8.5 dpc embryos cultured in each medium, the number which developed abnormalities in each medium, and whether these abnormlaities were major or minor. The major abnormalities observed in the cultured embryos were headfold defects, such as hydrocephaly; heart defects, such as an enlarged ventricle; yolk sac defects, including necrotic yolk sac layers; or necrosis of the embryo itself. The number of embryos with each of these defects in each medium is also noted.

9.25 and 9.5 dpc, and exhibited a smaller number of major abnormalities in comparison to the embryos cultured in DMEM : 50% dCP. (5/22 cf. 10/23) (Fig.14, Table 6). All 5 of the abnormal embryos exhibited necrosis, and 3 had an abnormal forebrain. This apparent difference between the capabilities of DMEM : 50% dCP and DMEM : 10% dCP to support *in vitro* development recapitulated the observation found with the 7.5 dpc embryo cultures.

In comparison, when 8.5 dpc embryos were cultured in FMP combined with either rat serum or dCP, their rate of development was as near normal as could be expected in an *in vitro* situation (Fig.12, 13, 14, Table 6). FMP : 50% rat serum, FMP : 10% rat serum, FMP : 50% dCP, and FMP : 10% dCP all supported development from 8.5 dpc to between 9.25 and 9.5 dpc. The only difference between the media was the number of abnormal embryos after 24 hours in culture (Fig.14, Table 6). There were no abnormalities found in any of the 27 embryos cultured in FMP : 50% rat serum, whereas 15 of the 101 embryos cultured in FMP : 10% rat serum showed some significant abnormality. All 15 of these abnormal embryos were necrotic, and 1 had an abnormal yolk sac. 32 embryos were cultured FMP : 50% dCP, and after 24 hours 1 embryo exhibited a degree of necrosis. This was the only abnormality seen with this culture medium. When the dCP was decreased to 10%, 4 out of 32 embryos were significantly abnormal. All of these embryos were necrotic.

To determine whether there was a statistically significant difference between the ability of the various media to support embryonic development from 8.5-9.5 dpc, the scores in each media were analysed in students t-tests. The results were similar to those for the 7.5 dpc embryos. A statistically significant difference was obtained when comparing FMP and DMEM-containing media, or DMEM plus serum with DMEM plus plasma (Appendix A2).

Statistical analysis of the data suggested that FMP combined with rat serum or dCP were the most suitable media in which to culture 8.5 dpc embryos. However, as demonstrated by CCL64 assay, only rat serum contains TGF-β, and therefore FMP : 50% dCP was more suitable for the requirements of this project. 10% rat serum and 10% dCP in combination with FMP also supported development, but both resulted in slightly more abnormalities.

3.3 In-Depth Analysis of 8.5 dpc Embryo Culture.

Analysis of the 8.5 dpc embryo cultures suggested that further investigation was warranted to examine the significant difference in development when embryos were cultured in FMP : 50% dCP or 10% dCP, and DMEM : 50% dCP. In a further twelve experiments, 30-32 embryos were cultured for 24 hours in each medium. The embryos were then assessed using a number of methods. As well as scoring the phenotype by the Brown-Fabro method (Brown & Fabro, 1981) (Table 3), and recording any abnormalities, the embryos were examined histologically; cell proliferation was measured by BrDU incorporation; and embryonic protein content was measured. These criteria enabled other aspects of embryonic development to be measured and compared, rather than just the morphological appearance.

To circumvent day-to day variation in culture conditions, in each experiment all three culture media were tested on litter mates.

3.3.1 Phenotypic Analysis of Embryos After Culture.

26 embryos cultured in DMEM : 50% dCP, 26 in FMP : 50% dCP, and 27 embryos cultured in FMP : 10% dCP were morphologically analysed to assess the ability of each medium to support development (Fig.15). The embryonic scores indicated that FMP : 50% dCP was more efficient than either FMP : 10% dCP or DMEM : 50% dCP, although FMP : 10% dCP was almost as effective, as had been found previously (Fig.12, 14, Table 6). However, when the abnormalities were classified (Table 7), FMP : 10% dCP was found to induce more abnormalities, with 8 out of 27 embryos exhibiting one or more major defect, in comparison to only 3 out of 26 embryos cultured in FMP : 50% dCP. The majority of abnormalities observed in the FMP : 10% dCP embryos were forebrain defects (6/8) such as anencephaly, or hydrocephaly (Table 7). The FMP : 50% dCP defects were equally distributed between the yolk sac (2/3), the forebrain (2/3), and necrosis (1/3). In comparison, 24 of the 26 embryos cultured in DMEM : 50% dCP exhibited a major defect. All 24 were necrotic, some of which had additional defects in the yolk sac, forebrain, or heart. The embryos appeared to have died early on in the culture period, as they were all 9 dpc or younger.

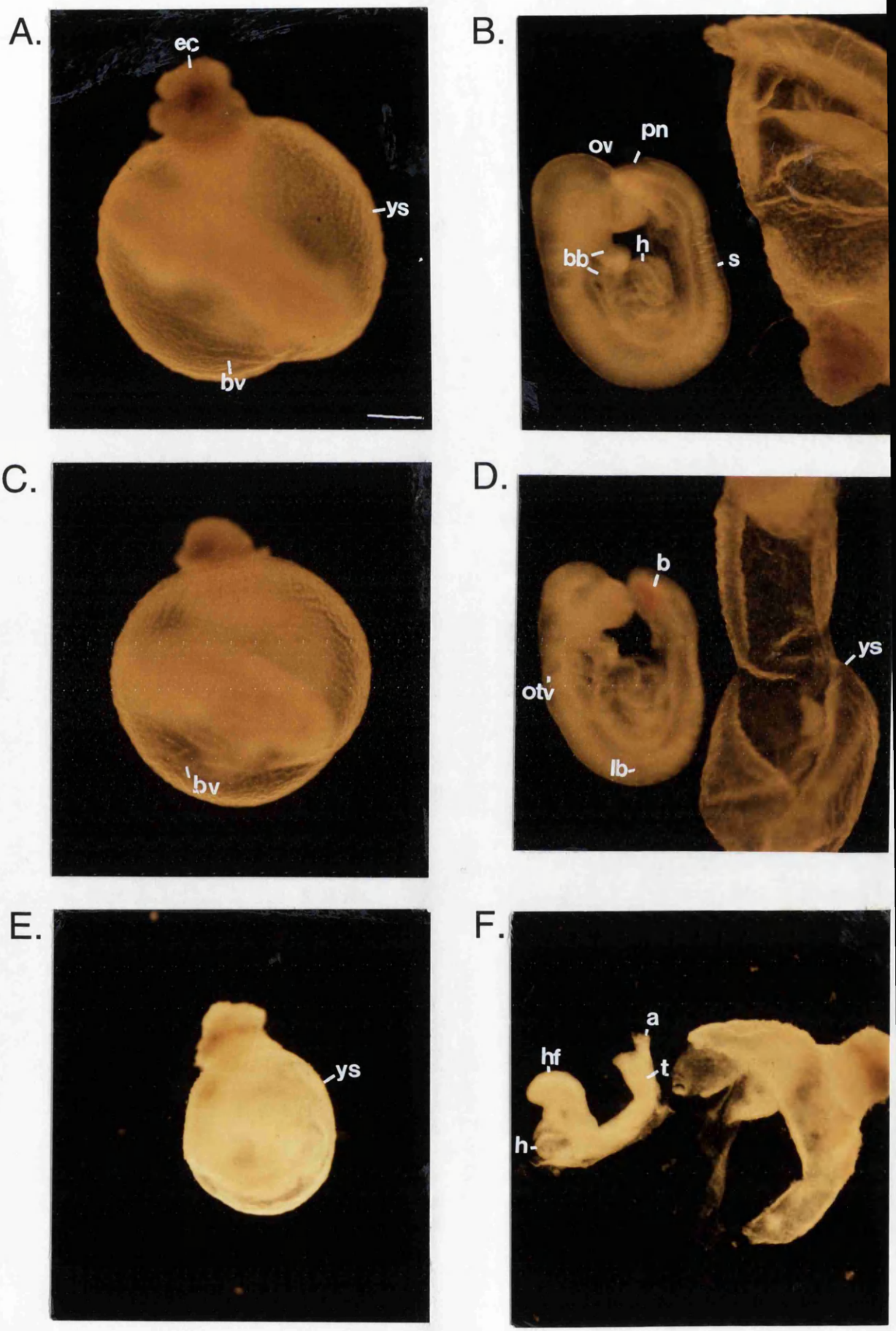
These results agreed with the preliminary data in 3.2.3. FMP : 50% dCP was the most efficient medium to culture embryos from 8.5 to 9.5 dpc, followed closely by FMP : 10% dCP. DMEM : 50% dCP however, was completely inefficient. Two-sample

Figure 15 - Further Analysis of 8.5 dpc Embryo Cultures.

Additional culture experiments were performed using FMP : 50% dCP, FMP : 10% dCP, and DMEM : 50% dCP. Embryos were removed from the uterus at 8.5 dpc, and cultured in one of the above media for 24 hours. (A) shows one such embryo with the yolk sac still intact after culture in FMP : 50% dCP. The yolk sac was removed to examine the morphology of the embryo proper (B). (C) and (D) show a FMP : 10% dCP cultured embryo, while the embryo in (E) and (F) was cultured in DMEM : 50% dCP.

a - allantois; b - blood; bb - branchial bars; bv - blood vessel; ec - ectoplacental cone; h - heart; hf - headfolds; lb - limb bud; otv - otic vesicle; ov - optic vesicle; pn - posterior neuropore; s - somites; t - tail; ys - yolk sac.

Scale bars : (A-F) - 560μm.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	Normal Development	Abnormal Development		Abnormal Yolk Sac	Abnormal Forebrain	Abnormal Heart	Necrotic
FMP : 50% dCP	23/26 (88%)	3/26 (12%)		3/3 (100%)	3/3 (100%)	0 (0%)	1/3 (33%)
FMP : 10% dCP	16/27 (70%)	8/27 (30%)		4/11 (50%)	8/11 (100%)	1/11 (12.5%)	6/8 (75%)
DMEM : 50% dCP	2/26 (8%)	24/26 (92%)		5/26 (21%)	6/26 (25%)	2/11 (8%)	24/26 (100%)

Table 7 - Abnormalities Observed during Indepth Study of 8.5 dpc Embryo Cultures.

On examination of the 8.5 dpc embryos cultured in FMP : 50% dCP, FMP : 10% dCP, or DMEM : 50% dCP, it was noted that there were a number of abnormalities after 24 hours in culture. 26 embryos were cultured in FMP : 50% dCP, and 27 in each of FMP : 10% dCP and DMEM : 50% dCP. The table above shows the number of normal versus abnormal embryos resulting from culture in each medium, and the distribution of the abnormal phenotypes. Defects were observed which affected the yolk sac, the heart, and the forebrain. The abnormalities were found in embryos cultured in each medium.

students t-tests demonstrated that there was a significant difference between the scores of the embryos cultured in the different media ($P \leq 0.05$ for all comparisons), providing statistical evidence to corroborate the morphological evidence (Appendix A3).

In comparison to the preliminary studies, there was a considerably higher number of abnormal embryos, for the DMEM : 50% dCP cultured embryos (Table 6, 7). In the initial studies, 10/23 embryos had major abnormalities, and 8/23 had minor abnormalities, whereas in this study, 24/26 had major abnormalities, and 0/26 had minor abnormalities. This may have been due to batch differences of dCP or DMEM. However, as the FMP plus dCP media did not show these vastly increased numbers of abnormal embryos, it is likely that the DMEM was at fault.

3.3.2 Histology and BrDU Labelling of Cultured Embryos.

Morphological analysis and scoring gives a broad overview of the development of the whole embryo, but does not assess the smaller details. The embryos were therefore labelled with BrDU for 1 hour and analysed histologically to examine the integrity of the cells, tissues, and internal structures, and to estimate DNA synthetic capacity.

Initial difficulties were encountered when trying to administer the BrDU. Four methods were tried and tested; culturing the embryos with the yolk sac intact in medium containing BrDU; culturing embryos with the yolk sac opened; injecting BrDU intraperitoneally into the pregnant female; and injecting BrDU into the yolk sac cavity of cultured embryos.

Initially seventeen 9.5 dpc embryos were cultured with the yolk sac intact in FMP : 50% dCP + 1µl 10mmol/l BrDU for 2-2.5 hours. Once they were sectioned and stained, BrDU was seen to have incorporated into the nuclei of the ectoplacental cone but not into any of the yolk sac or embryonic cells. Therefore, the next method attempted was to apply the BrDU to the embryos *in vivo,* based on the idea that the BrDU would cross from the mothers blood into the embryo. 500µl of 10mmol/l BrDU was injected intraperitoneally into a 30g pregnant female when the embryos were approximately 9.5 dpc. 3 females were treated in this way, and left for 1.5, 5 and 6.5 hours to allow for transfer of the BrDU, before a schedule 1 kill to obtain the embryos. 21 embryos were obtained from these experiments, but, again, no staining was observed in the embryo or yolk sac, only in the ectoplacental cone. The third attempt returned to *in vitro* methods, but this time the embryos were cultured with their yolk sac open for 1.5 hours in the presence of 1µl of 10mmol/l BrDU. This

method was more successful with staining observed in the yolk sac cells, and in sporadic regions of the embryo. However, this latter staining appeared to be artefactual, and suggested that perhaps the BrDU was too dilute in the 1ml of medium. Therefore, because the BrDU was ineffective in 1ml of medium, and that, from the intraperitoneal and intact yolk sac culture methods, it appeared as if the BrDU was unable to cross the yolk sac, 1µl of 10mmol/l BrDU was injected directly into the yolk sac cavity using a pulled micropipette, and the embryos cultured for 1 hour in FMP : 50% dCP. This method enabled the BrDU to incorporate into the DNA of the cells of the yolk sac and the embryo, and therefore the experimental analysis of the cultured embryos could begin.

Seven 8.5 dpc embryos were cultured in FMP : 50% dCP, six in FMP : 10% dCP, and six in DMEM : 50% dCP for 23 hours before the injection of 1µl of 10mmol/l BrDU into their yolk sac cavity. They were then cultured for a further hour to allow incorporation of the BrDU, before fixing in 4% PFA, and processing to sections. 3 additional embryos were removed from the uterus at 9.5 dpc and injected with BrDU before culturing for 1 hour in FMP : 50% dCP. These embryos were controls against which the cultured embryos could be compared. An immunohistochemical staining procedure revealed the cells which had incorporated the BrDU (Materials and Methods), enabling histology and the rates of cell proliferation to be compared between the cultured embryos, and with the controls.

Analysis of the control embryo sections enabled normal histology to be observed (Fig.16A). By visualisation, there appeared to be slightly more cells in the control sections in comparison with the sections from cultured embryos (Fig.16B-D), but this could have been due to a difference in embryonic age. As mentioned in section 3.3.1, delayed development was observed in the cultured embryos, especially those cultured in DMEM : 50% dCP. The histology of the embryos cultured in either of the FMP-containing media was very similar to that of the controls, with very few pyknotic cells, and no evidence of hyper- or hypoplasia (Fig.16, 17). The only difference seen in a few sections of the FMP cultured embryos was that the mesenchymal cells appeared to be slightly less densely packed, possibly indicative of oedema. All other cell types were normal. The sections of the DMEM : 50% dCP cultured embryos clearly illustrated the lack of growth in these embryos, and their demise. Many of the cells were pyknotic, and there was very little development of the different tissues and organs (Fig.16, 17).

The results of the histological analysis agreed with the previous observations that embryos cultured in FMP : 50% dCP and FMP : 10% dCP embryos grow almost as

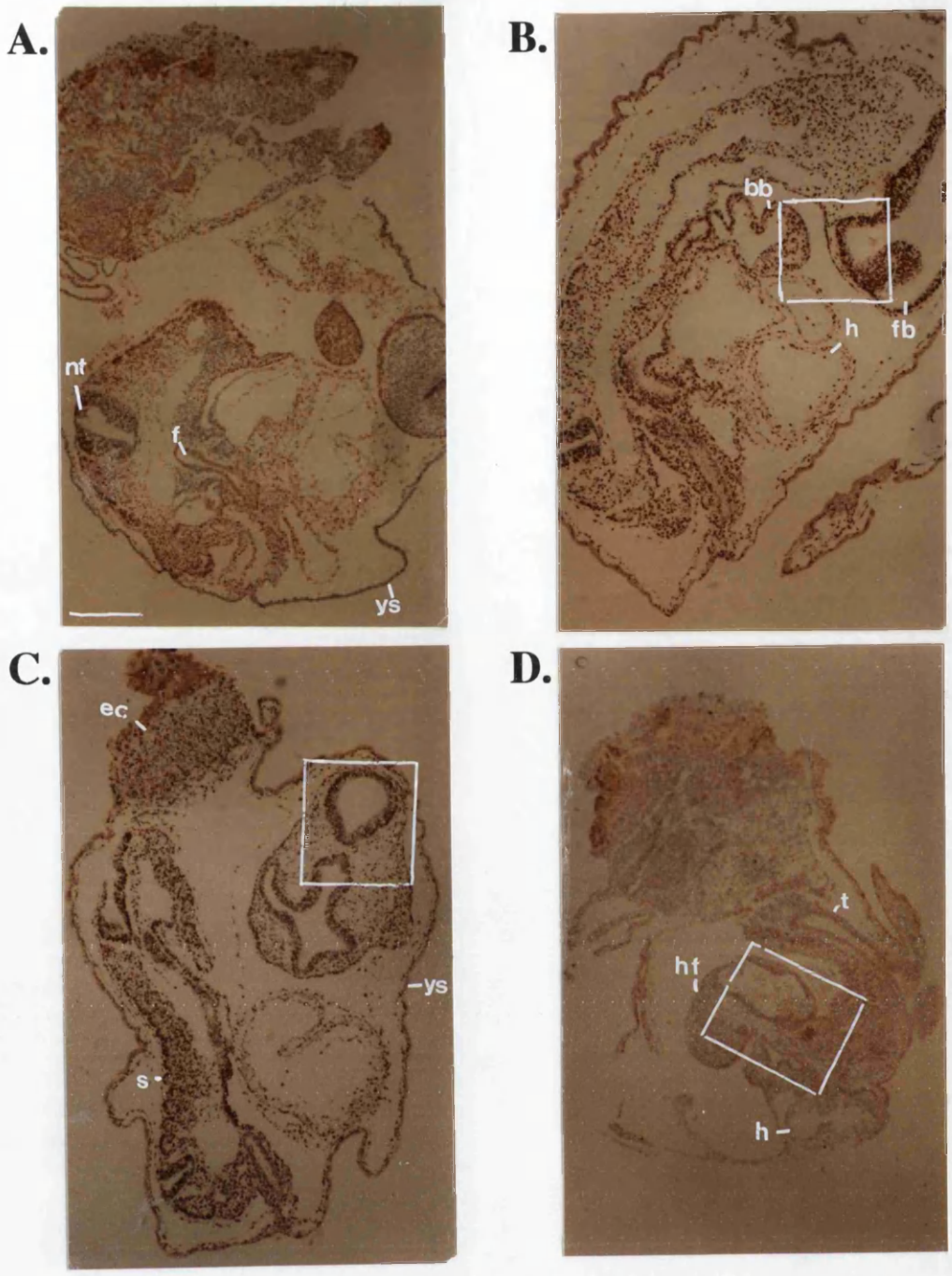


Figure 16 - Histological Analysis of 8.5 dpc Cultured Embryos.

BrDU stained sections of cultured embryos were analysed histologically. (A) is a section through a control 9.5 dpc embryo. (B), (C), and (D) were all cultured embryos. (B) - FMP : 50% dCP; (C) - FMP : 10% dCP; (D) - DMEM : 50% dCP. Note the severely delayed development, and the high amount of necrosis (shown in more detail in Fig.17) in (D) in comparison to any of the other examples.

bb - branchial bars; ec - ectoplacental cone; f - foregut; fb - forebrain; h - heart; hf - headfolds; nt - neural tube; s - somites; t - tail; ys - yolk sac.

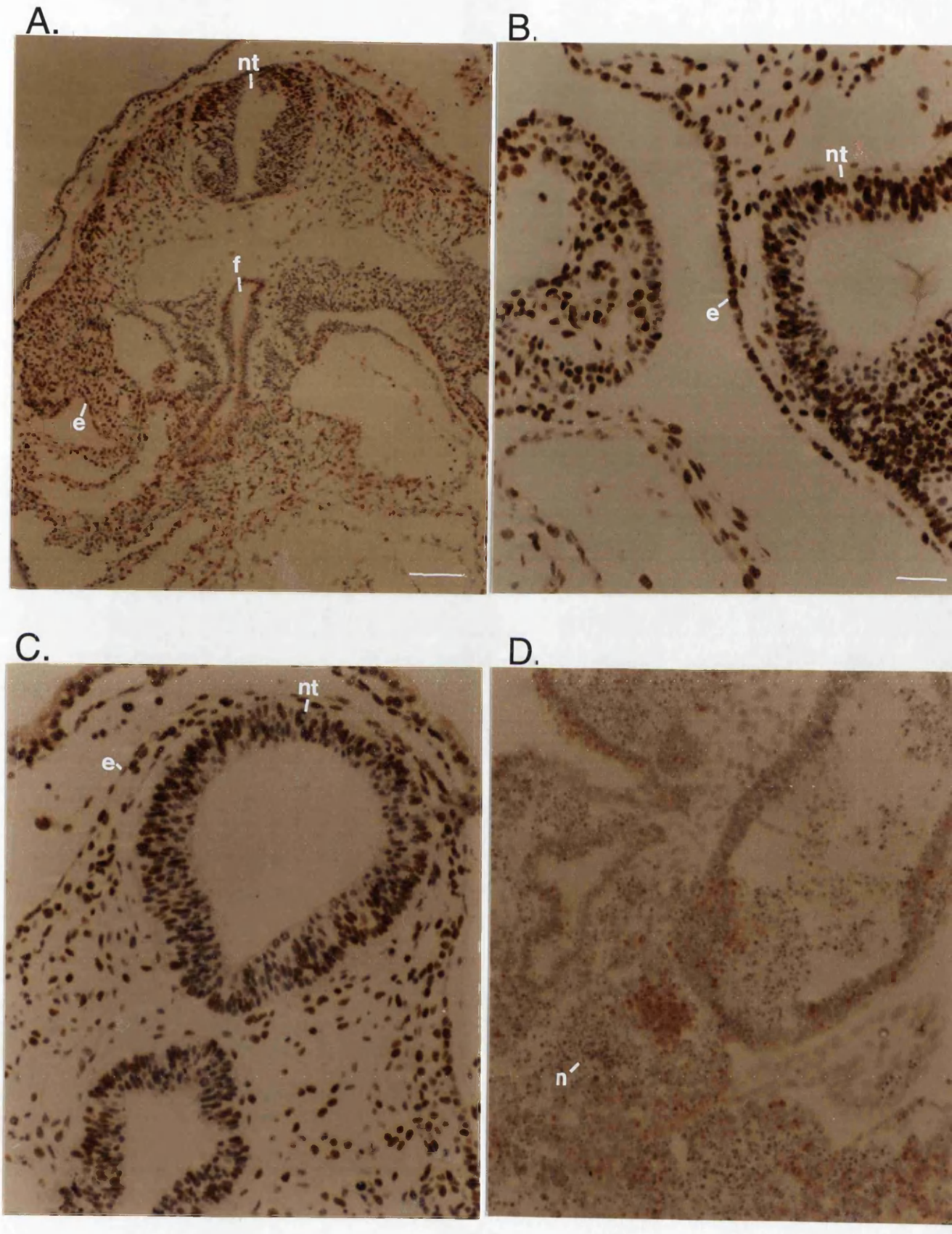
Scale bars : (A-D) - 375μm.

Figure 17 - Cell Proliferation in Control and Cultured Embryos.

Control or cultured embryos were subjected to BrDU incorporation in order to examine and compare cellular proliferation rates. The ectodermal cells were analysed by counting the number of BrDU stained cells (brown) versus the number of non-stained cells (blue). (A) control embryo with a high level of cell proliferation, especially in structures near the ectodermal surface of the embryo. At higher magnification, the embryos cultured in FMP : 50% dCP (B), or FMP : 10% dCP (C) were also observed to have a high rate of proliferation. Embryos cultured in DMEM : 50% dCP (D) had little BrDU staining as the majority of the cells were necrotic, indicated by the small dark spots. (B), (C), and (D) are high magnification photographs of the boxed areas in Fig. 16 (B, C, D).

e - ectoderm; f - foregut; n - necrotic cells; nt - neural tube.

Scale bars - (A) - 150μm ; (B,C,D) - 75μm.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well as their *in utero* counterparts. As yet a system that is as efficient as the *in vivo* situation has not yet been found, therefore *in vitro* development will not be as precise as *in utero*. The histology of the DMEM : 50% dCP embryos also reflected the morphological analysis at a more detailed level, supporting the observation that this medium cannot support the development of 8.5 dpc embryos.

On initial examination of the DNA synthetic rate, sections of embryos that had been cultured in the same medium appeared to have comparable amounts of staining (Fig.16). Two embryos from each medium were then chosen for more detailed examination by the integrity of their tissues (Fig.17). By visualisation alone, the degree of staining suggested that the fraction of proliferating cells in embryos cultured in FMP : 50% dCP, FMP : 10% dCP and controls was very similar, whereas the fraction was much less in the DMEM : 50% dCP cultured embryos. There were also many pyknotic cells in the DMEM : 50% dCP sections, indicated by highly condensed, darkly stained nuclei, the histological manifestation of the necrosis observed morphologically (Fig.17).

To compare proliferative rates in the three media a total of 1000 epithelial cells per embryo were counted, and the number of stained and unstained cells noted (Fig.18, Appendix A4). The ectodermal cells which surround the embryo were chosen as they were abundant and easy to identify. An average of 39% of cells stained for BrDU in the FMP : 50% dCP cultured embryos, 40% in FMP : 10% dCP, 22% in DMEM : 50% dCP, and 32% in the controls. When the data was statistically analysed by a two-sample student t-test, the results indicated that there was a significant difference in cell proliferation rates between most of the different samples ($P \leq 0.05$) (Appendix A4). However, only the DMEM : 50% dCP embryos had significantly reduced levels of BrDU incorporation. Embryos cultured in either of the media containing FMP exhibited higher proliferation rates than the controls. It is possible that the controls exhibited a reduced number of proliferating cells in comparison to the FMP-cultured embryos due to the labelling procedure. Cultured embryos had been in the medium for 23 hours before the addition of BrDU, whereas the control embryos were labelled directly after dissection, and may have been in shock.

3.3.3 Protein Analysis of Cultured Embryos.

In contrast to the phenotype of an embryo which does not change in a simple quantitative manner over time, the amount of protein in an embryo increases logarithmically with age. Protein analysis therefore gives a reliable estimation of embryonic development because it is possible to affect the mass of an embryo without

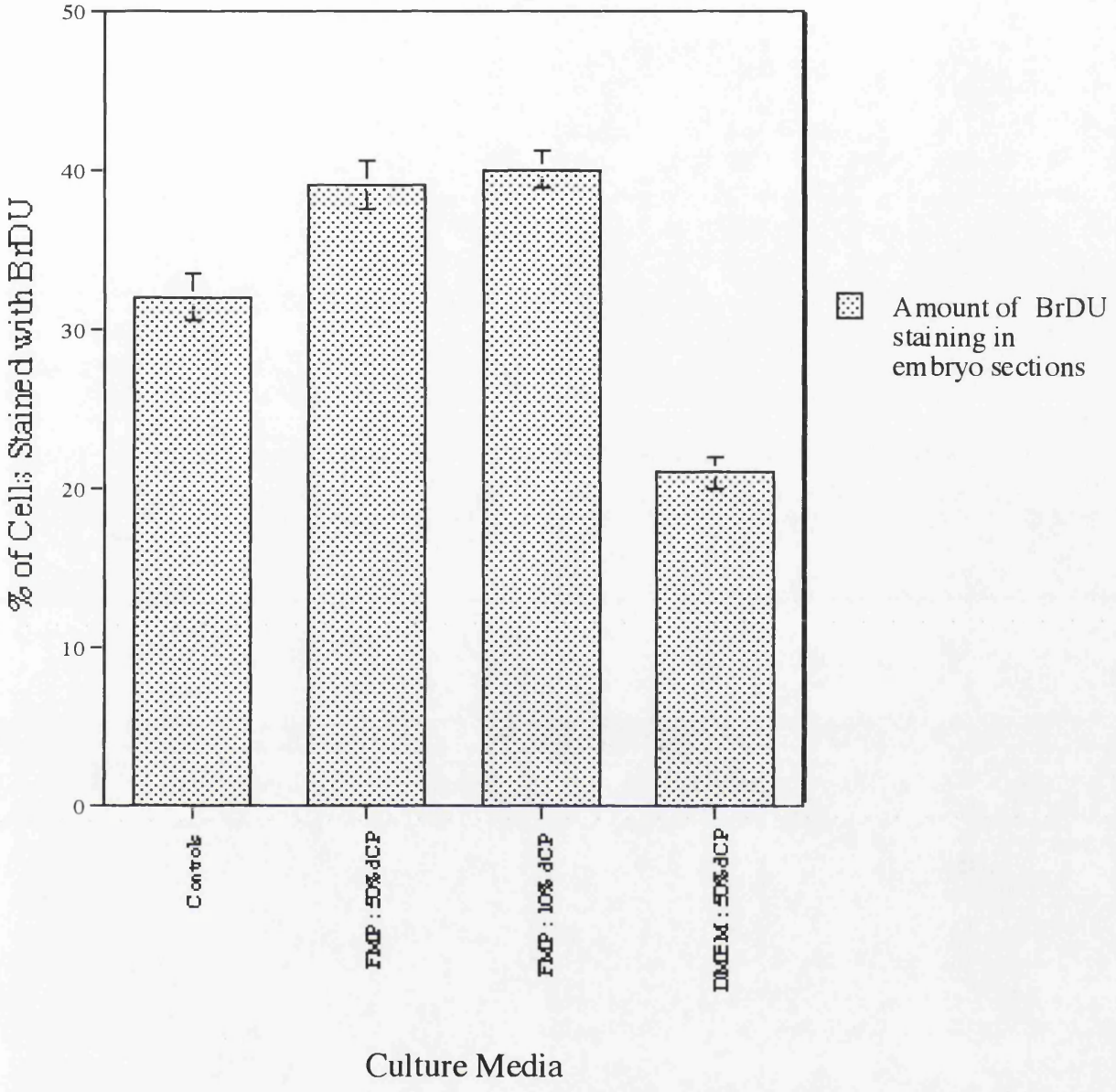


Figure 18 - BrDU Incorporation in Cultured Embryos as a Measurement of Cell Proliferation.

Culturing embryos were injected with 1µl of 10mmol/l BrDU to analyse growth and development in the different media. BrDU incorporation in the ectodermal cells of 2 embryos from each medium, and two control embryos was measured, thereby assessing cell proliferation. A total of 2000 cells were counted in each pair of embryos, and the percentage of cells that were stained with BrDU is shown on the histogram above . The vertical bars represent the standard error.

severely affecting the appearance. Therefore, to further analyse the difference in embryonic development in each of the media, the protein content of individual embryos was measured. Protein extraction and estimation was performed using the Lowry method from 15 embryos cultured in FMP : 50% dCP, 17 in FMP : 10% dCP, 16 in DMEM : 50% dCP, and 6 uncultured, control 9.5 dpc embryos (Lowry *et al*, 1951) (Materials and Methods). The cultured embryos were obtained from 5 different experiments, and frozen at -20°C until required. All protein samples were extracted concurrently, and measured individually, in triplicate, in a spectrophotometer. The embryos in each group exhibited a wide range of protein values:- FMP : 50% dCP=126-482; FMP : 10% dCP=84-338; DMEM : 50% dCP=109-259; controls=159-574 μg/embryo (Fig.19). Due to the manner by which the protein was extracted, this variation could not be attributed to accidental loss during the extraction procedure, and so therefore, it had to reflect the variation in the size or developmental stage that each embryo had attained. When the protein values were compared with the embryo scores, the reason for the variation was confirmed. Embryos with a high score had a high protein concentration, while those with a low score had a lower protein concentration (Fig 20).

When the protein data was analysed statistically using a two-sample 2-sample students t-test, there was evidence for a significant difference ($P \leq 0.05$) in the protein content of embryos cultured in the FMP-containing media, in comparison to the embryos cultured in DMEM : 50% dCP (Appendix A5). When FMP : 50% dCP and FMP : 10% dCP were compared, the P-value was 0.17, and therefore not statistically significant. However, when DMEM : 50% dCP was compared with FMP : 50% dCP, the P-value was 0.0024, and with FMP : 10% dCP, $P = 0.0079$. These values represent a highly statistically significant difference. When the protein levels of the cultured embryos were compared with controls, only the DMEM : 50% dCP showed a significant difference (Appendix A5). The protein levels of embryos cultured in FMP : 50% dCP or FMP : 10% dCP were comparable with control embryos.

3.3.4 Conclusions.

The results of the above investigations demonstrated that there was a significant difference between the different media in their ability to support the development of 8.5 dpc embryos in culture. FMP : 50% dCP (<5pg/ml TGF-β1) was the most efficient at maintaining the growth of the embryos, although FMP : 10% dCP (<1pg/ml TGF-β1) was almost as effective. DMEM : 50% dCP did not support development, and often resulted in the death of the embryos. Embryos that were cultured in the FMP media were very similar to control embryos that had not been

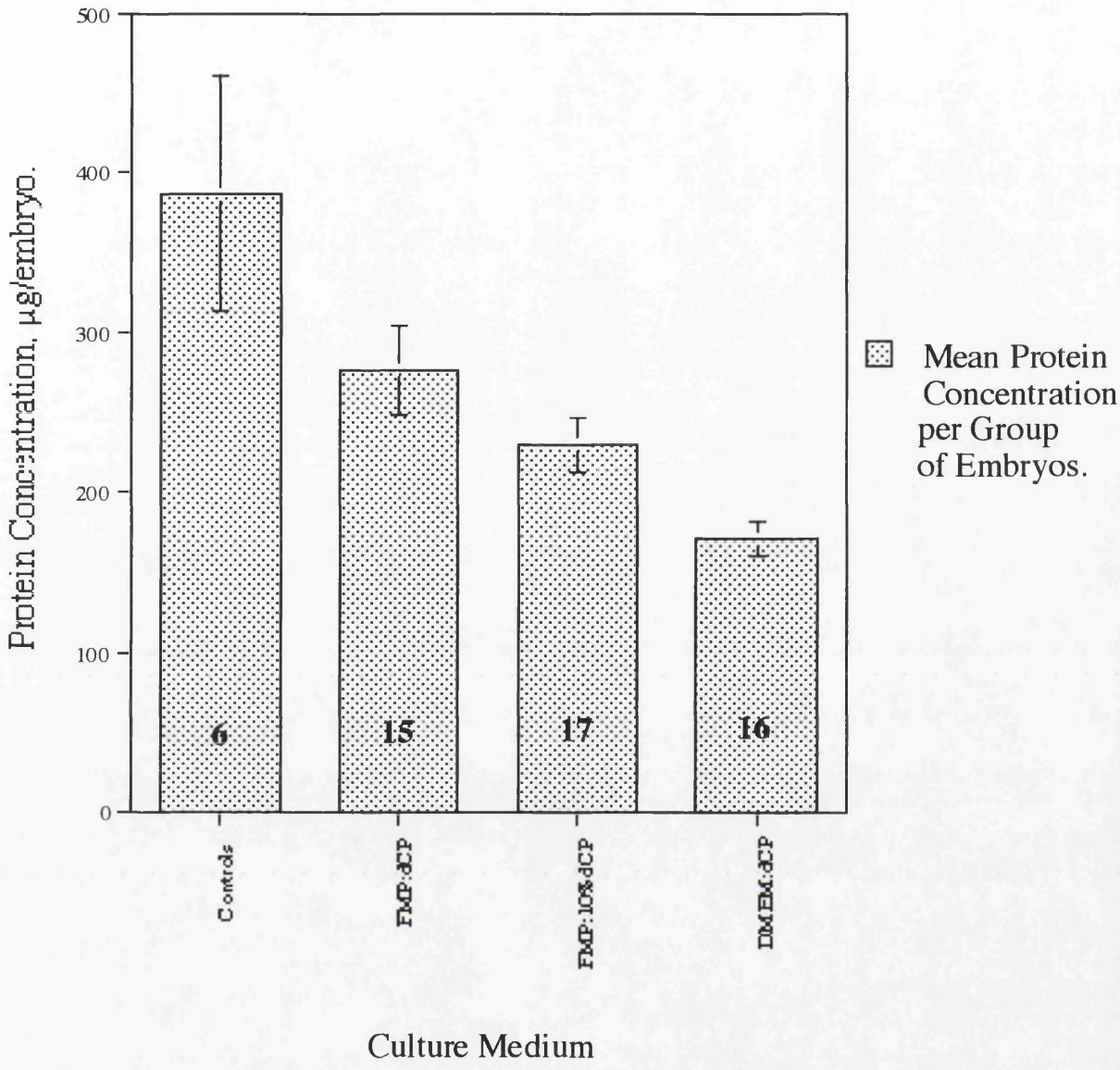


Figure 19 - Concentration of Protein in Cultured Embryos.

The protein was extracted from embryos cultured in different media, or control embryos to assess their development. Each embryo was treated separately, and the protein extracted, then measured in triplicate by the Lowry method (Lowry, 1951). The mean protein concentration for the embryos in each group is represented on the histogram. The number of embryos analysed is shown by the figure in each columns. The vertical bars represent the standard error of the mean.

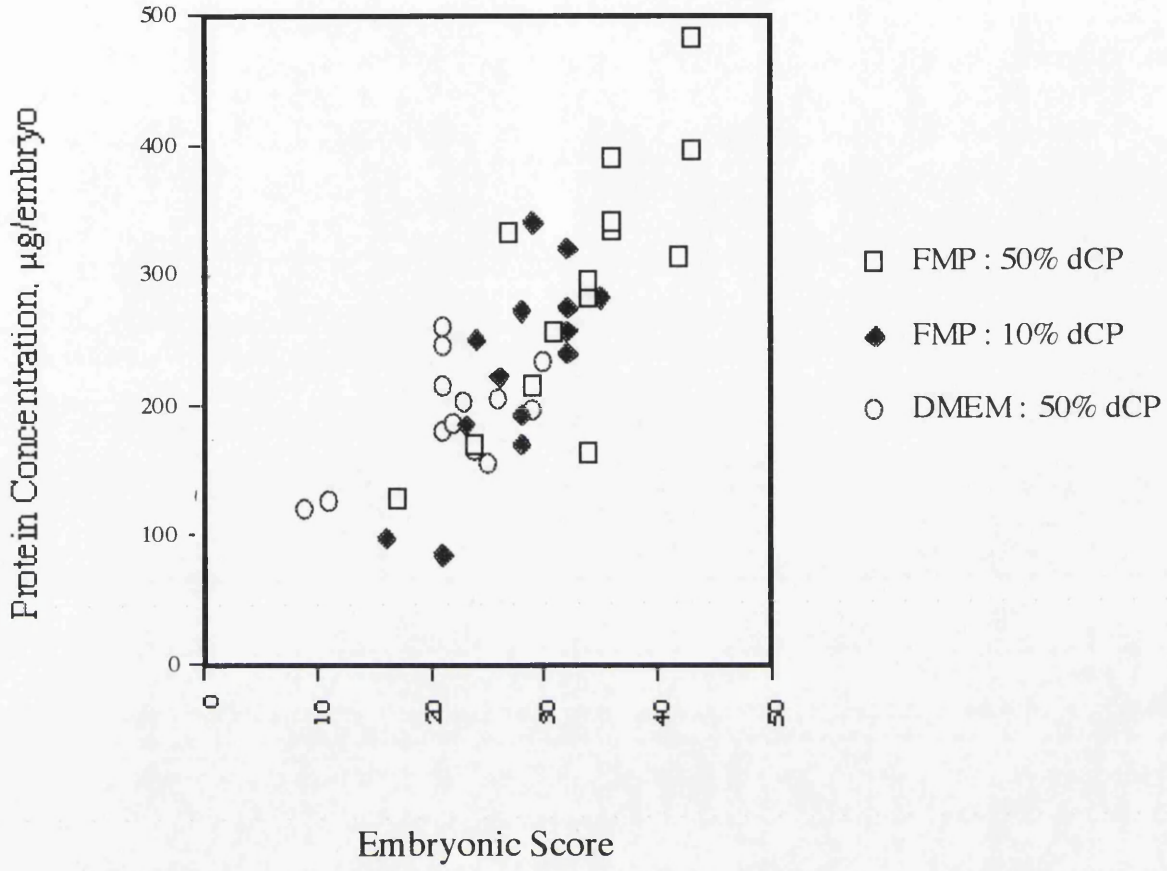


Figure 20 - Comparison of Embryonic Score and Protein Concentration.

To determine if the embryonic variation in protein concentration reflected the variation in developmental stage of the embryos after culture, the embryo scores were compared with the protein concentrations. Each individual point on the graph above represents an embryo, with embryos cultured in the different media distinguished by different symbols (see key). As can be seen, with an increase in embryonic score, there was a corresponding increase in protein concentration.

cultured; in cell proliferation rate, histology, and protein content, and only on morphological scoring was there a significant difference. This is most likely as a result of the nature of the scoring system. There can be a relatively large difference in the scores of two embryos if one is only slightly more advanced than the other. By incorporating the other investigative techniques, more reliable results were obtained. In conclusion, a TGF-β1-depleted medium was identified (FMP : 50% dCP) which could support complete development of the embryo *in vitro* from 8.5 to 9.5 dpc.

3.4 Developmental Analysis of Embryos Which Lack TGF-β1.

Two independent laboratories generated TGF-β1 knockout mice using homologous recombination techniques (Shull *et al*, 1992; Kulkarni *et al*, 1993). Both groups found that 50% of the TGF-$\beta1^{-/-}$, and 25% of the TGF-$\beta1^{+/-}$ offspring died pre- or peri-natally. These mice were available to us on a mixed genetic background (50% NIH, 37.5% C57Bl6, 12.5% 129sv), so we endeavoured to analyse the developmental abnormalities which led to the death of the TGF-β1 knockout embryos. This work was performed in close liason with Dr. Marion Dickson, who determined, by genotype analysis of embryos from intercrosses of TGF-$\beta1^{+/-}$ mice, that the embryos died at 9.5 to 11.5 dpc (Dickson *et al*, 1995). Independently from, but in collaboration with Dr. Dickson, the current project set out to a) characterise the nature of the defects appearing *in utero*, and b) to assess whether the defects were amplified in a medium lacking TGF-β1. The reason for this latter objective was that one study had suggested that a proportion of the TGF-β1 knockout embryos were rescued by maternal TGF-β1 (Letterio *et al*, 1994).

3.4.1 Genotype Analysis of TGF-β1 Knockout Embryos.

Since it was known that some of the TGF-β1 knockout embryos died around 10.5 days gestation, analysis was performed on embryos of 8.5 and 9.5 dpc. TGF-$\beta1^{+/-}$ x TGF-$\beta1^{+/-}$ matings were set up, and the embryos harvested into PBS or Embryo Transfer Freezing Medium. After morphological analysis each embryo was digested with proteinase K, and the genomic DNA genotyped by PCR analysis utilising two sets of oligonucleotide primers as described in Materials and Methods (Fig.3, Table 4).

One problem encountered during the genotype analysis was that embryonic DNA was not as easy to amplify as adult tail DNA, requiring a further 5 PCR cycles in addition to the 30 required for tail DNA. There was also a higher failure rate with the embryonic DNA. PCR using tail DNA failed approximately 5% of the time, whereas with embryonic DNA, it failed approximately 15-20% of the time (Fig.21). As well as there being less DNA in the embryonic samples, it is possible that as embryos have many different types of proteins expressed, and the tail is predominantly keratin, the embryonic proteins may bind to the DNA, and therefore interfere with the PCR. If the PCR failed, the DNA was cleaned by a further phenol-chloroform extraction to remove any extra protein, before repeating the reaction. Contamination of the samples was a very rare occurrence when amplifying tail DNA, but again, occurred slightly

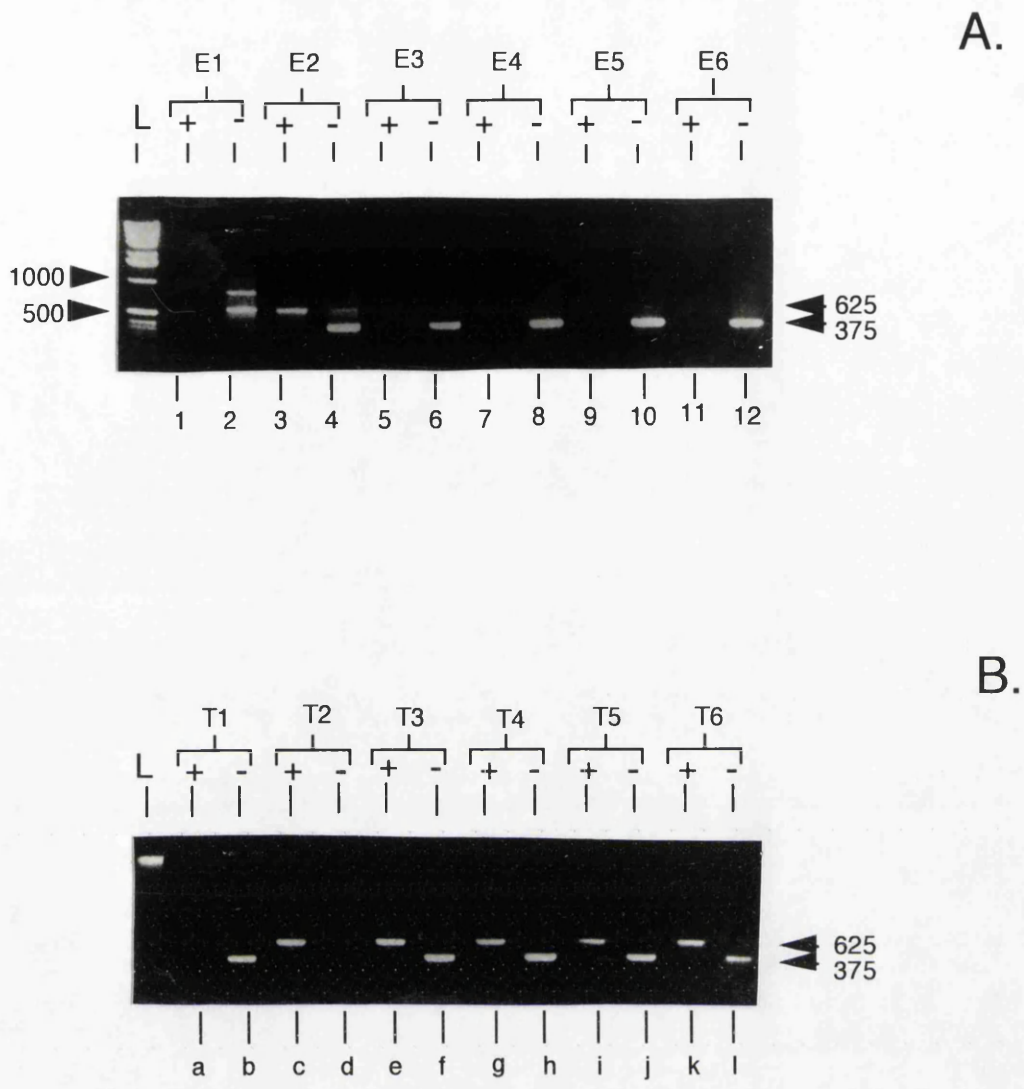


Figure 21 - Genotype Analysis of TGF-β1 Knockout Animals by PCR.

DNA extracted from embryo sample (A) or tails (B) was genotyped by PCR. Each individual lane represents a separate PCR reaction, amplifying either the wild-type TGF-β1 allele (+), or the null allele (-). The product for the + allele was 625bp, while the - allele was 375bp. (A) - Lanes 2 and 4 show exceptional contaminant bands, although embryo 2 (E2) was genotyped as TGF-$\beta 1^{+/-}$ as the correct sized band was present and intense. The PCR for Embryo 1 (E1) was repeated. As can be seen in (B), there was no contamination for the tail DNA PCR.

L - 1kb Ladder.

more often with embryonic DNA. As shown in Fig.21A (lane 2), spurious band contamination did occur, but this was extremely rare. More common was the detection of the bands for both the null and normal alleles in a single PCR product (Fig.21, lane 4). This should not have occurred due to the fact that the alleles were amplified separately in different eppendorf tubes, as described in Materials and Methods. This observation suggested that there was primer contamination, although it is also possible that there was leakage of the PCR products in the adjacent lane while loading the gel. However, contamination of either type was usually not a problem as the correct sized bands were commonly of a much stronger intensity than the contaminants. However, if the results were dubious, then the PCR was repeated.

3.4.2 Analysis of Embryos After Development *in vivo.*

To determine whether it was possible to recognise the TGF-$\beta1^{+/-}$ and TGF-$\beta1^{-/-}$ embryos that would die at 9.5-11.5 dpc, 8.5 dpc embryos were first to be examined for abnormalities. 93 embryos were collected from TGF-$\beta1^{+/-}$ intercrosses, and analysed phenotypically. All embryos and their corresponding yolk sacs were normal, regardless of genotype. Therefore, the embryos destined to die were not morphologically discernable at this stage.

Following the lack of abnormal phenotypes in the 8.5 dpc embryos, one hundred and sixty-four 9.5 dpc embryos from TGF-$\beta1^{+/-}$ x TGF-$\beta1^{+/-}$ matings were examined. Analysis of the morphological appearance of the conceptuses revealed that, although all of the embryos *per se* were normal, there were a number of yolk sacs exhibiting abnormal phenotypes (Fig.22, Table 8). These abnormalities specifically affected the vasculature and haematopoietic systems. To determine whether these defects were restricted to TGF-$\beta1^{+/-}$ and TGF-$\beta1^{-/-}$ yolk sacs, and therefore represented the initial phenotypic effect of ablation of TGF-$\beta1$, 1/2 of each embryo was used for genotype analysis. Of the 164 embryos collected, 156 were successfully genotyped, revealing that there were 40 TGF-$\beta1^{+/+}$, 78 TGF-$\beta1^{+/-}$, and 38 TGF-$\beta1^{-/-}$ knockout embryos; the expected Mendalian ratio of 1:2:1. Conclusive genotypes could not be obtained for the remaining 8 embryos. When the phenotypes and genotypes were compared, 17 out of 78 TGF-$\beta1^{+/-}$ yolk sacs, and 23 of 38 TGF-$\beta1^{-/-}$ yolk sacs were abnormal (Table 8). These values represent a ratio of 1:1.7:0.4 for the number of normal yolk sacs in each genotype, and corresponds with the 25% and 50% loss of TGF-$\beta1^{+/-}$ and TGF-$\beta1^{-/-}$ embryos, respectively (expected 1:1.5:0.5 cf. observed 1:1.7:0.4, P=0.18). This indicates that an abnormal yolk sac was probably responsible for embryonic lethality.

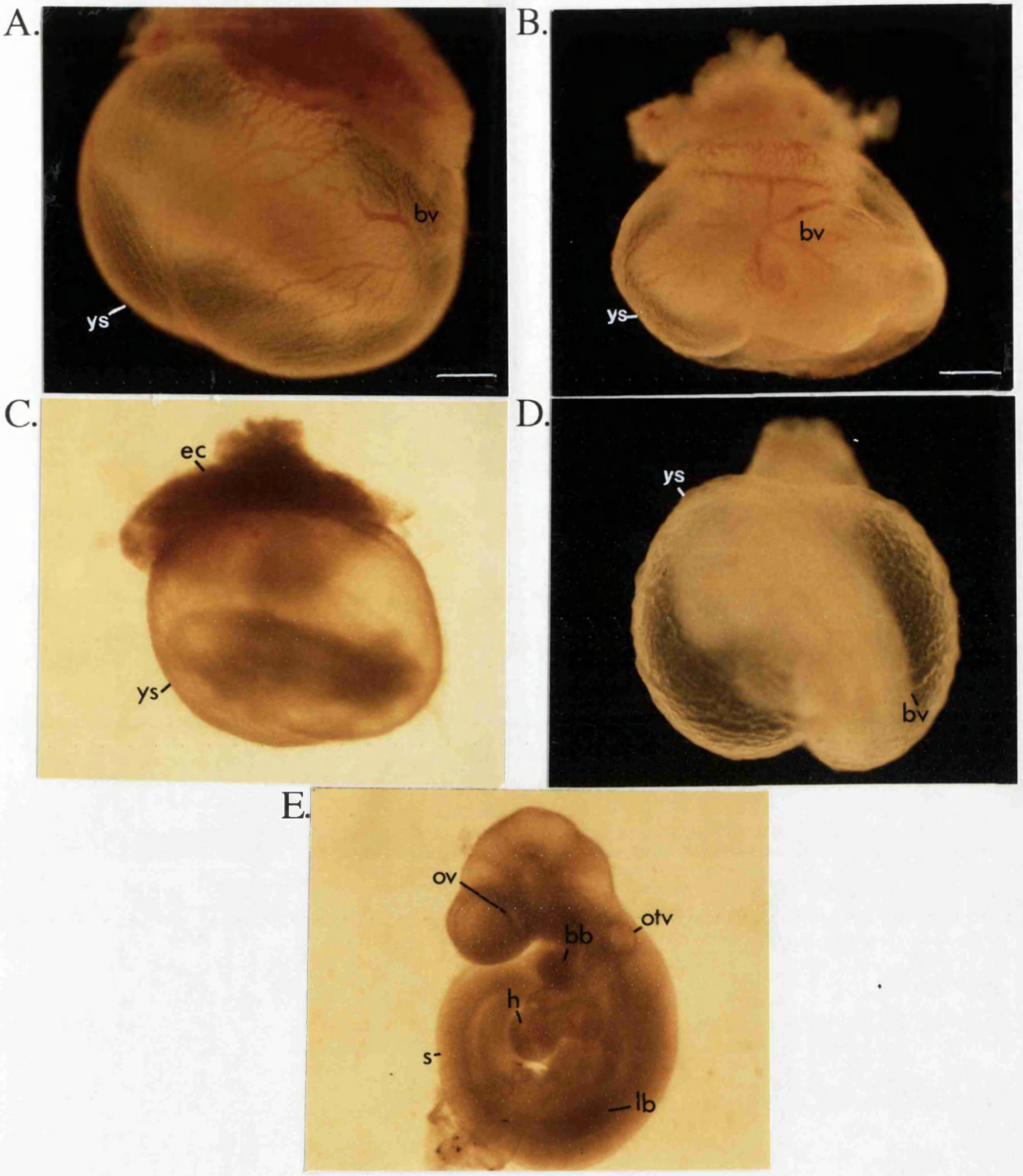


Figure 22 - Morphological Analysis of TGF-β1 Knockout Embryos.

9.5 dpc embryos from TGF-$\beta1^{+/-}$ intercrosses were removed from the uterus and examined morphologically. (A) TGF-$\beta1^{+/-}$ conceptus with normal yolk sac vasculature and haemoglobinisation. (B) TGF-$\beta1^{-/-}$ conceptuses with disorganised vasculature, and (C) no vessels or blood. (D) TGF-$\beta1^{+/-}$ yolk sac with a well-developed vasculature, but severe anaemia. (E) Morphologically normal TGF-$\beta1^{-/-}$ embryo. At 9.5-9.75 dpc, the embryos were usually phenotypically normal, regardless of the phenotype of the yolk sac.

a - allantois; bb - branchial bars; bv - blood vessel; ec - ectoplacental cone; h - heart; lb - limb bud; otv - otic vesicle; ov - optic vesicle; s - somites; ys - yolk sac.

Scale bars : (A,D) - 600μm ; (B,C,E) - 560μm.

	TGF-β1$^{+/+}$	TGF-β1$^{+/-}$	TGF-β1$^{-/-}$
Full Yolk Sac Plexus	32 (80%)	58 (8 Anaemic) (74%)	14 (1 Anaemic) (37%)
Developing Yolk Sac Plexus	5 (12%)	3 (4%)	1 (3%)
Delicate/ Disorganised Vessels	3 (8%)	14 (2 Anaemic) (18%)	14 (6 Anaemic) (37%)
No Vessels		3 (1 Anaemic) (4%)	9 (6 Anaemic) (24%)
Total	40	78	38

Table 8 - Yolk Sac Phenotypes Observed In TGF-β1 Knockout Embryos.

156 embryos from TGF-β1$^{+/-}$ intercrosses were removed from the uterus at 9.5 dpc and morphologically analysed. A number of abnormal yolk sac phenotypes were observed, although the embryos *per se* were normal. After genotype analysis, the distribution of the yolk sac phenotypes in the TGF-β1$^{+/+}$, TGF-β1$^{+/-}$, and TGF-β1$^{-/-}$ embryos was identified. The defects and their relationship to genotype are shown in the table above.

A variety of abnormal vascular and haematopoietic phenotypes were observed in both the TGF-β1$^{+/-}$ and TGF-β1$^{-/-}$ yolk sacs. The normal yolk sac at 9.5 dpc has an extensive, although not yet fully mature vasculature, which contains red blood cells (Fig.22). In contrast, many of the abnormal TGF-β1$^{+/-}$ and TGF-β1$^{-/-}$ yolk sacs had a reduced number of vessels, which often appeared to be delicate and disorganised (Fig.22). This was often accompanied by the blood leaking out of the vessels, either into the yolk sac cavity, or between the endodermal and mesothelial cell layers throughout the yolk sac. However, the most severe phenotype was when the yolk sac appeared to have developed normally to approximately 8.5-9 dpc, but had failed to undergo vasculogenesis, with the result that, although blood islands and an extending corona were present, no actual blood vessels had formed (Table 8).

Anaemia was also observed in the abnormal TGF-β1$^{+/-}$ and TGF-β1$^{-/-}$ yolk sacs, either in association with, or separately from, the vasculature defect (Fig.22, Table 8). Some yolk sacs exhibited both defects, while others had a normal vasculature, but lacked red blood cells, or conversely, had many red blood cells but no vessels in which to contain them (Fig.22). The severity of the anaemia also varied, from the presence of pale blood, to a lack of red blood cells altogether.

Combining the phenotypic and genotypic data indicated that the abnormal yolk sac phenotypes were restricted to the TGF-β1$^{+/-}$ and TGF-β1$^{-/-}$ yolk sacs, and were therefore likely to be as a result of reduced levels of TGF-β1. 3 out of 40 TGF-β1$^{+/+}$ embryos did appear to have delicate vessels, but the blood was still contained within the vessels, and showed no leakage as observed in the TGF-β$^{+/-}$ and TGF-β1$^{-/-}$ yolk sacs. The other 37 TGF-β1$^{+/+}$ yolk sacs all had extended, strong blood vessel systems, which contained red blood.

3.4.3 Post-Implantation *in vitro* Culture of TGF-β1 Knockout Embryos.

Although the abnormalities observed in the embryos *in vivo* were restricted to the TGF-β1$^{+/-}$ and TGF-β1$^{-/-}$ yolk sacs, some TGF-β1$^{+/-}$ and TGF-β1$^{-/-}$ yolk sacs were normal (Fig.22). Letterio *et al* (1994) suggested that a percentage of the embryos with reduced levels of TGF-β1 may be rescued by maternal TGF-β1. Therefore, to test this theory, embryos from TGF-β1$^{+/-}$ x TGF-β1$^{+/-}$ matings were removed at 8.5 dpc, before any defects were detectable, and cultured in FMP : 50% dCP for 24 hours. This was performed to determine if a greater percentage of the embryos developed the yolk sac phenotypes when removed from the exogenous maternal source of TGF-β1.

93 embryos, from 13 matings, were cultured separately in 1ml of medium to avoid any possible rescue of TGF-$\beta1^{+/-}$ or TGF-$\beta1^{-/-}$ embryos by their TGF-$\beta1^{+/+}$ siblings. After culture, the embryos and yolk sacs were morphologically assessed, before 1/2 of each embryo was used for genotyping, and the rest was fixed in 4% PFA, and stored for reference, or for histological analysis.

3.4.3.1 Morphological Analysis of Cultured Embryos.

Despite the absence of defects when the 8.5 dpc embryos were placed in culture, the phenotypes observed after *in vitro* culture were the same as those observed *in vivo*, namely a reduced number of vessels, delicate vessels, or no vessels, with or without a degree of anaemia (Fig.23, Table 9). These defects were, again, mainly restricted to the TGF-$\beta1^{+/-}$ and TGF-$\beta1^{-/-}$ yolk sacs, although 4 of the 34 TGF-$\beta1^{+/+}$ yolk sacs did have delicate vessels, and in one of these the blood had leaked into the yolk sac cavity. However, 8 out of 37 TGF-$\beta1^{+/-}$ embryos had an abnormal yolk sac; 5 with delicate or disorganised vasculature, and 3 were completely devoid of vessels. Of the 22 cultured TGF-$\beta1^{-/-}$ embryos, there were 6 yolk sacs with delicate or disorganised vessels, and another 5 with no vessels at all. As for the *in vivo* study, anaemia was only observed in TGF-$\beta1^{+/-}$ or TGF-$\beta1^{-/-}$ yolk sacs, regardless of the presence or absence of vasculature defects (Table 9). In general, all embryos *per se,* independent of the genotype, were phenotypically normal after 24 hours in culture. However, when the yolk sac vasculature developed no further than the blood island stage, development of the embryo was correspondingly delayed (Fig.23).

To determine if there was an increase in the number of abnormalities in the absence of maternal TGF-β1, the number of defective yolk sacs in *in vivo* (Table 8) and in cultured embryos (Table 9) was compared. There was an identical number of abnormal TGF-$\beta1^{+/-}$ yolk sacs in both studies (22%), whereas there were fewer defective TGF-$\beta1^{-/-}$ yolk sacs in the *in vitro* study (50% cf. 60%). Therefore, when embryos were removed from maternal TGF-β1 and cultured *in vitro*, the number of abnormal yolk sacs did not increase. However, these results do not dismiss the possibility that maternal TGF-β1 is rescuing a percentage of the embryos, but rather suggests that if it does transpire, then it does so before 8.5 dpc.

3.4.3.2 Histological Examination of Cultured Embryos.

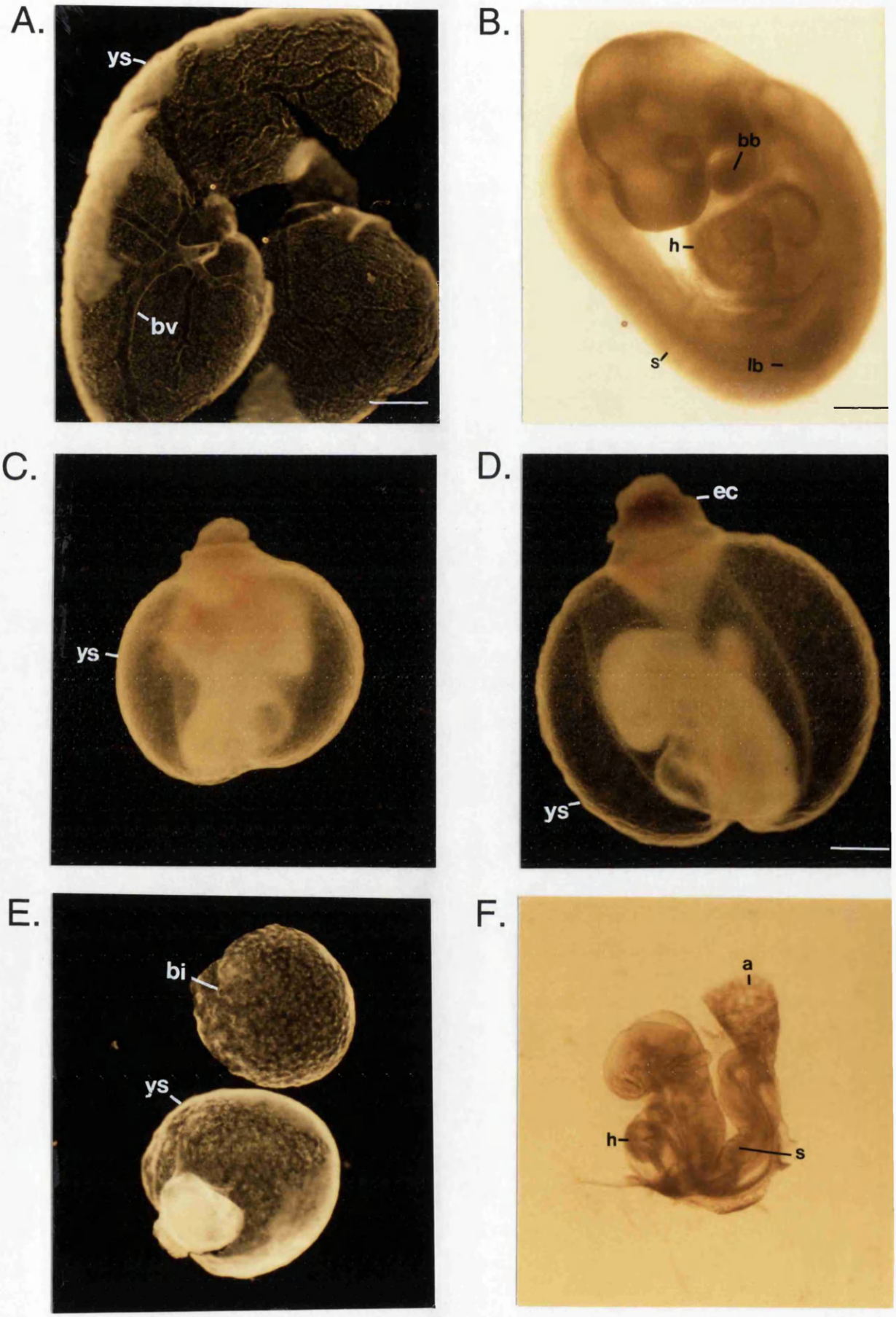
For a closer examination of the yolk sacs and embryos, 10 of the 9.5 dpc TGF-β1 knockout embryos which had been examined morphologically, were also sectioned, and analysed histologically. 3 TGF-$\beta1^{+/+}$ and 7 TGF-$\beta1^{-/-}$ embryos were examined.

Figure 23 - Morphological Analysis of Cultured TGF-β1 Knockout Embryos.

8.5 dpc embryos from TGF-$\beta 1^{+/-}$ intercrosses were removed from the uterus and cultured in FMP : 50% dCP for 24 hours. They were morphologically analysed after culture, followed by genotypic analysis. (A) TGF-$\beta 1^{+/+}$ yolk sac with well-developed vasculature, and (B) its 9.5 dpc embryo. (C) TGF-$\beta 1^{+/-}$ conceptus with a few vessels which appear delicate. The blood has leaked into the yolk sac cavity. (D) TGF-$\beta 1^{-/-}$ yolk sac with no vessels or blood, although there is a little blood in the embryo proper. (E) TGF-$\beta 1^{-/-}$ yolk sac with persistent blood islands indicating the delayed development of the yolk sac vasculature, and (F) delayed embryo dissected out from the yolk sac in (E).

a - allantois; bb - branchial bars; bi - blood island; bv - blood vessel; ec - ectoplacental cone; h - heart; lb - limb bud; s - somites; ys - yolk sac.

Scale bars : (A,C,E) - 560μm ; (B,F) - 375μm ; (D) - 450μm.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	TGF-β1$^{+/+}$	TGF-β1$^{+/-}$	TGF-β1$^{-/-}$
Full Yolk Sac Plexus	21 (61.8%)	25 (2 Anaemic) (67.6%)	7 (31.8%)
Developing Yolk-Sac Plexus	6 (17.6%)	4 (10.8%)	4 (18.2%)
Delicate/ Disorganised Vessels	6 (17.6%)	5 (1 Anaemic) (20%)	6 (4 Anaemic) (27%)
No Vessels		3 (12%)	5 (3 Anaemic) (23%)
Total	33	37	22

Table 9 - Yolk Sac Phenotypes after *In Vitro* Culture.

92 embryos from TGF-β1$^{+/-}$ intercrosses were removed from the uterus at 8.5 dpc, and cultured for 24 hours in FMP : 50% dCP, before morphological analysis. These cultures were performed to determine whether the abnormal phenotype observed in the *in vivo* embryos would be accentuated in embryos removed from maternal TGF-β1. An increase in the incidence of abnormal yolk sac phenotypes was not observed after culture, with the same range of defects as observed *in vivo*. Again, the embryos *per se* were normal. Genotype analysis identified the distribution of the yolk sac phenotypes in the TGF-β1$^{+/+}$, TGF-β1$^{+/-}$, and TGF-β1$^{-/-}$ conceptuses, and the defects and their relationship to genotype are shown in the table above.

All 3 TGF-$\beta 1^{+/+}$, and 3 of the TGF-$\beta 1^{-/-}$ yolk sacs were morphologically normal, while the other 4 TGF-$\beta 1^{-/-}$ yolk sacs exhibited abnormal phenotypes. For comparison, three 9.5 dpc NIH/Parkes embryos were also analysed. Some of the sections were stained with H&E, while others were utilised for BrDU analysis.

In agreement with the morphological study, the histology of the embryos *per se* was normal, regardless of genotype. However, variations were observed in the yolk sacs (Fig.24, 25). The mesothelial and endodermal layers of the NIH/Parkes yolk sacs were closely apposed, except in small regions where they were separated by the endothelial cells of a blood vessel. 2 of the 3 TGF-$\beta 1^{+/+}$ yolk sacs, and all 3 of the phenotypically normal TGF-$\beta 1^{-/-}$ yolk sacs also exhibited this close apposition. However, all of the sections of the abnormal TGF-$\beta 1^{-/-}$ samples exhibited shearing of the yolk sac layers, especially in regions where the endoderm was buckled. The buckling probably occurred as a result of the embedding process, and was found in embryo sections of all genotypes. There were also large regions where the yolk sac was not wrinkly, but the layers had separated regardless. When the histological and morphological data were compared, these large gaps were found specifically in the yolk sacs with delicate vessels in which the blood had leaked out and flowed free between the endoderm and mesothelial cell layers.

The data above suggested that the connections between the different cell types were weakened by the lack of TGF-$\beta 1$, either directly or indirectly. Some of the cell connections may have been relatively strong, but not strong enough to withstand the wrinkling of the yolk sac during embedding, whereas the observation of sheared regions in areas where there was no wrinkling suggests that some of the cell connections had already broken *in vivo*. Therefore, the TGF-$\beta 1^{+/-}$ and TGF-$\beta 1^{-/-}$ embryos that survive gestation may do so because the cell connections in these yolk sacs remain strong, and keep the different cell layers closely apposed.

Analysis of the morphological and histological data for the abnormal TGF-$\beta 1^{+/+}$ yolk sac indicated that this yolk sac was slightly underdeveloped in comparison to the other samples. It is therefore possible that the cell connections were also underdeveloped, with the result that the cell layers sheared during the embedding process.

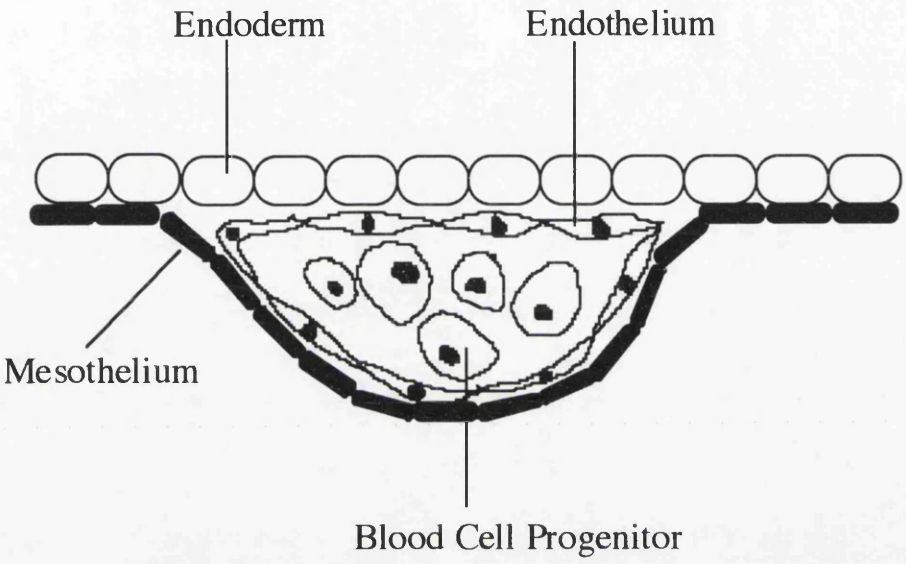

Figure 24 - The Layers of the Yolk Sac of a 9-9.5 dpc Mouse Embryo.

Schematic diagram of the 9-9.5 dpc yolk sac in cross section. The three cell layers of the yolk sac are shown, and labelled, with the blood progenitor cells within the vessel.

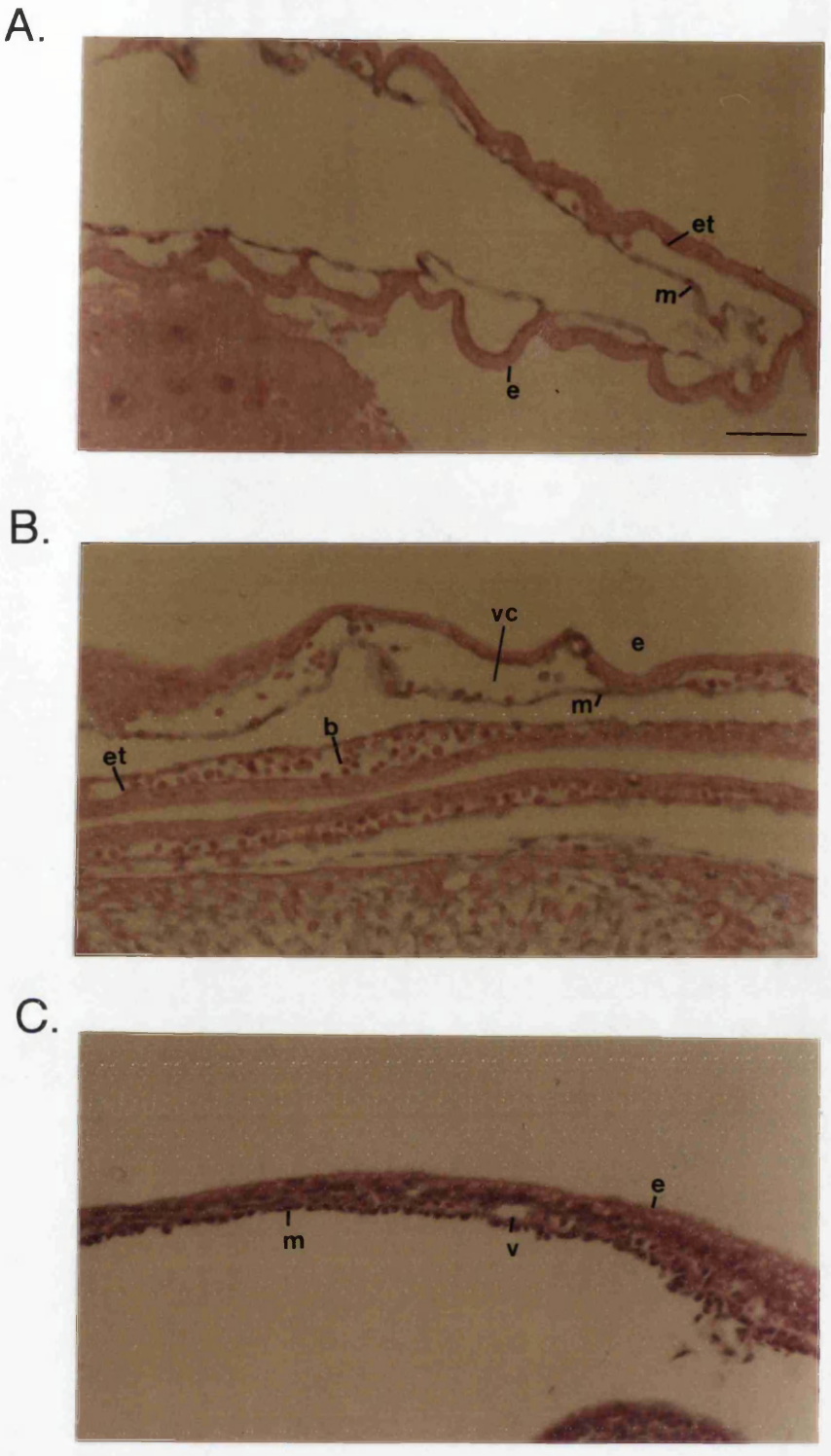

Figure 25 - Histological Analysis of 9.5 dpc Yolk Sacs.

The structure of the yolk sacs in the TGF-β1 knockout conceptuses was analysed from haematoxylin and eosin stained sections. (C) TGF-$\beta1^{+/+}$ yolk sac with the mesothelial and endodermal layers closely apposed, except where there is a vessel. (A) An abnormal TGF-$\beta1^{-/-}$ yolk sac where the layers are not closely apposed, and the endoderm has buckled. (B) TGF-$\beta1^{-/-}$ yolk sac in which the layers have separated without buckling of the endoderm, and created very large vascular channels

b - blood cell; e - endodermal cell; et - endothelial cell; m - mesothelial cell; v - vessel; vc - vascular channels.

Scale bars : (A,B,C) -75μm.

3.4.4 Bromo-DeoxyUridine (BrDU) Labelling of TGF-β1 Knockout Embryos.

From *in vitro* studies, TGF-β1 has always been considered to be an inhibitor of cell proliferation (Moses *et al*, 1985; Ohta *et al*, 1987). It would therefore be expected that depletion of the protein would result in increased proliferation. However, by morphological analysis, neither the TGF-$\beta1^{+/-}$ or TGF-$\beta1^{-/-}$ embryos or yolk sacs appeared hyperplastic. To verify this, BrDU labelling was utilised to examine the cell proliferation rates. 36 embryos from 5 TGF-$\beta1^{+/-}$ x TGF-$\beta1^{+/-}$ matings were removed from the uterus at 9.5 dpc, and 1μl of 10mmol/l BrDU was injected directly into the yolk sac cavity using a pulled micropipette. The embryos were then cultured for 1 hour in FMP : 50% dCP, enabling the BrDU to be incorporated. After culture, they were analysed phenotypically before half of the embryo was removed for genotyping, and the remainder of the embryo plus yolk sac was processed for sectioning.

The rate of DNA synthesis in the cells of the yolk sac, and in two types of epithelial cells in the embryo was analysed in 3 normal TGF-$\beta1^{+/+}$ and 3 phenotypic TGF-$\beta1^{-/-}$ samples.

3.4.4.1 Yolk Sac Cells.

At 9.5 dpc the yolk sac consists of three different cell types (Fig.24). The endodermal cell layer is the outer layer of the yolk sac. This is closely apposed to the mesothelial cell layer, which lines the yolk sac cavity. In certain regions of the yolk sac, this close apposition is interrupted by the presence of endothelial cells forming the vessels of the yolk sac (Fig.26). To examine the cell proliferation rate in the yolk sac, all of these cell types were initially counted together, with approximately 1000 cells counted per yolk sac (ie. 3000 per genotype). On comparison of the number of BrDU stained cells in the different genotypes, it was noted that the proliferation rates of TGF-$\beta1^{+/+}$ and TGF-$\beta1^{-/-}$ yolk sacs were not significantly different (Fig.27, Appendix A6). However, counting all of the cells together may have masked a phenotypic difference in only one cell type. Therefore, it was decided to examine each cell type individually.

Approximately 1500-3000 endodermal, mesothelial and endothelial, or endothelial cells alone were counted in the TGF-$\beta1^{+/+}$ and the TGF-$\beta1^{-/-}$ yolk sacs (Fig.26, 27, Appendix A6). The number of cells stained for BrDU in each cell type was compared between the genotypes using a two-sample students t-test. The results indicated that there was no significant difference ($P \leq 0.05$) in the rate of cell proliferation in any of

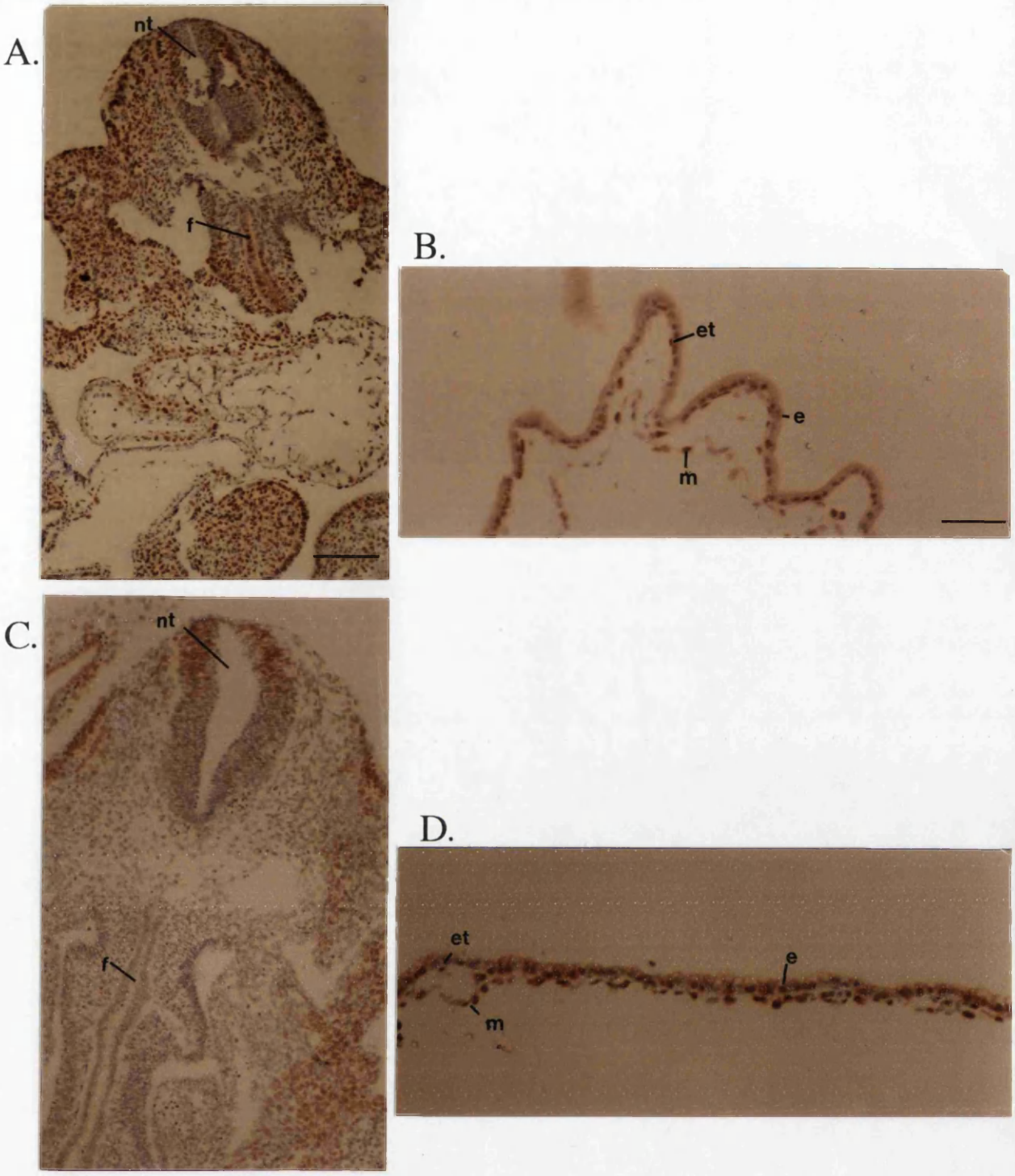

Figure 26 - BrDU Incorporation in TGF-β1 Knockout Conceptuses.

Cell proliferation rates were examined in TGF-$\beta1^{+/+}$ and TGF-$\beta1^{-/-}$ embryos and yolk sacs using BrDU incorporation and staining techniques. The ectoderm and the neural tube epithelia in the embryo proper, and all three cell types in the yolk sac were analysed. (A) TGF-$\beta1^{+/+}$ embryo showing extensive staining in many tissue types, which was also observed in the TGF-$\beta1^{-/-}$ embryo (C). The TGF-$\beta1^{+/+}$ (D) and TGF-$\beta1^{-/-}$ (B) yolk sacs also had comparable amounts of staining.

e - ectodermal cell; et - endothelial cell; f - foregut; m - mesothelial cell; nt - neural tube.

Scale bars : (A,C) - 150μm ; (B,D) - 75μm.

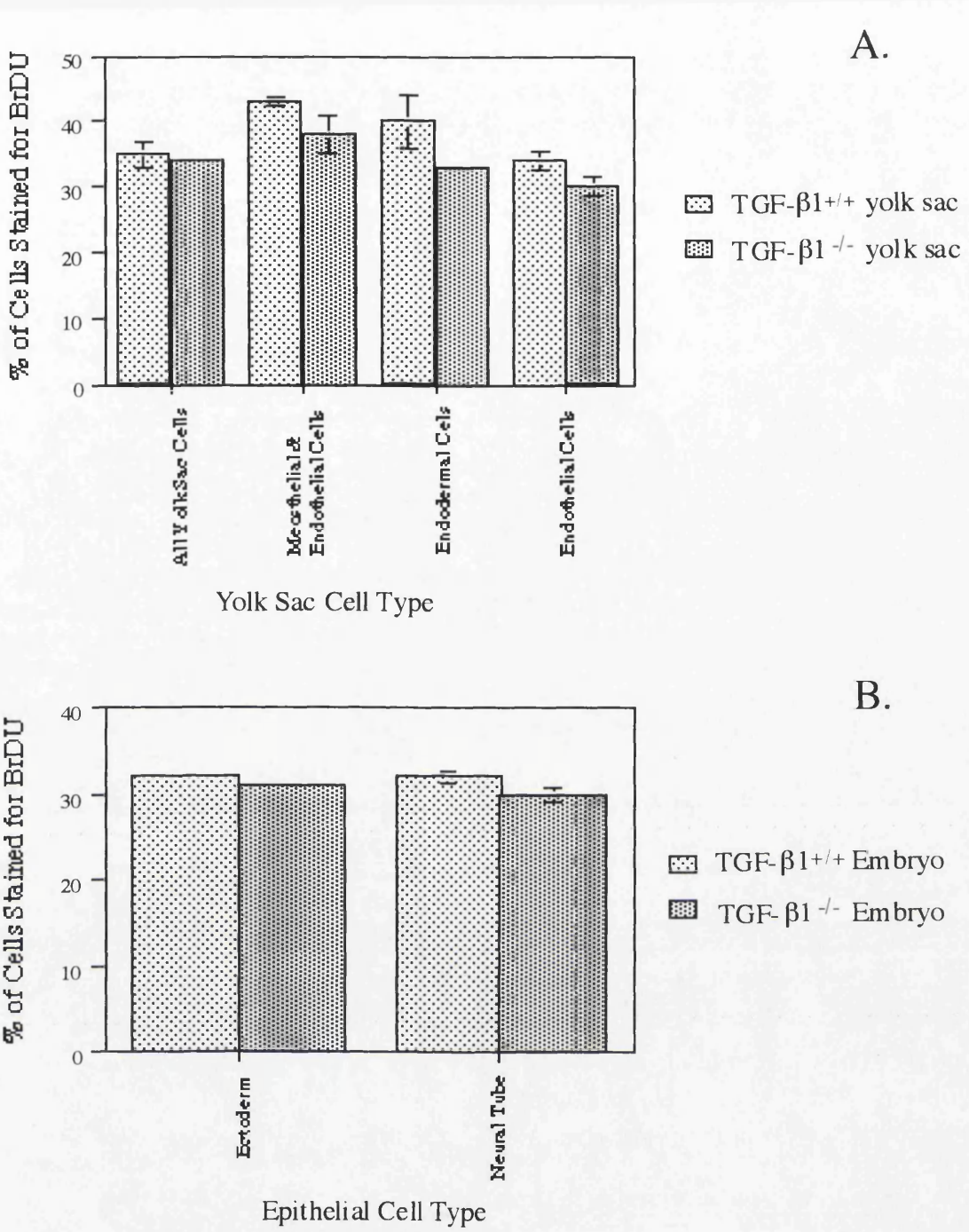

Figure 27 - Cell Proliferation Rate in TGF-β1$^{+/+}$ and TGF-β1$^{-/-}$ Embryos And Yolk Sacs.

To determine whether there was a difference in the cell proliferation rate in the TGF-β1$^{-/-}$ embryos due to the ablation of a potent growth inhibitor, the DNA synthetic rate was measured by the incorporation of BrDU. The number of stained cells in the embryo or yolk sac was compared between 3 TGF-β1$^{+/+}$ and 3 TGF-β1$^{-/-}$ samples. In the yolk sac (A), the cells were analysed in 4 different ways - by counting all cell types together, the mesothelial and endothelial cells together, the endodermal cells alone, or the endothelial cells alone. In the embryo (B), two types of epithelial cells were examined - the surrounding ectodermal cells, and the neural tube epithelial cells. Approximately 3000 cells were counted per cell type per genotype.The columns in the graph show the mean percentage of stained cells from the 3 embryos of each genotype. The standard error of the mean is shown by the vertical bars.

the three cell types in TGF-$\beta1^{+/+}$ and TGF-$\beta1^{-/-}$ yolk sacs (Appendix A6). This was especially important for the endothelial cells as it suggests that although these were the cells affected in the TGF-$\beta1^{-/-}$ yolk sacs with defective vasculature, the phenotype was not due to abnormal cell proliferation.

The above BrDU data suggests that TGF-β1 does not have any growth inhibitory action on any of the yolk sac cell types at this stage of development. Statistically, the data for the TGF-$\beta1^{+/+}$ and TGF-$\beta1^{-/-}$ yolk sacs was not significantly different, and in all of the cell types examined there was actually slightly less proliferation in the TGF-$\beta1^{-/-}$ yolk sacs than in the TGF-$\beta1^{+/+}$ yolk sacs. The BrDU analysis therefore coincides with the morphological data, and shows that hyperplasia of the yolk sac does not occur in the absence of TGF-β1. It also indicates that the defective phenotypes are not a result of abnormal cell proliferation.

3.4.4.2 The Embryo Proper.

As was found for the yolk sacs, hyperplasia was not observed in any of the embryos when they were examined morphologically or histologically, regardless of genotype, or phenotype of its corresponding yolk sac. To support this, cellular proliferation was assessed by BrDU incorporation in TGF-$\beta1^{+/+}$ and TGF-$\beta1^{-/-}$ embryos. Two epithelial cell types were examined, namely the ectoderm cells surrounding the embryo, and the neural tube epithelia (Fig. 26, 27). For each of the 3 TGF-$\beta1^{+/+}$, and the 3 TGF-$\beta1^{-/-}$ embryos, 1000 cells of each type were counted, and the results analysed statistically (Appendix A7). There was no significant difference in proliferation rates of either of the cell types between the two genotypes.

These results agree with the descriptive analysis for the lack of hyperplasia in the phenotypic TGF-$\beta1^{-/-}$ embryos, and again asks whether TGF-β1 has an growth inhibitory role in the developing embryo. It may be, however, that TGF-β1 has an inhibitory effect in the embryo at a later stage in development.

3.4.5 Culture of TGF-β1 Knockout Embryos in FMP : Rat Serum.

As mentioned previously (section 3.2.1), rat serum contains a substantial amount of TGF-β1. In connection with the hypothesis that maternal TGF-β1 rescues a percentage of abnormal TGF-β1 knockout embryos *in utero* (Letterio *et al*, 1994), this project examined whether serum TGF-β1 could rescue the embryos destined to die. Seventy-four 8.5 dpc embryos from 9 TGF-$\beta1^{+/-}$ x TGF-$\beta1^{+/-}$ matings were cultured in FMP : 50% rat serum for 24 hours before morphological analysis. 19 control

Parkes/NIH embryos from 4 litters were simultaneously cultured in FMP : 50% rat serum.

The results were surprising (Table 10). Contrary to the rat serum having a beneficial effect on the embryos, it actually induced more abnormalities (Fig.28). After culture, 46 of the 75 embryos were defective (Table 10). The abnormal embryonic phenotypes included stunted development, hydrocephaly, anencephaly, and severe necrosis. 48 of the 75 yolk sacs were also abnormal (Fig.28, Table 10); the phenotypes including disorganised vessels, bubbles in the yolk sac, and a reduced number of vessels. These abnormalities were observed in conceptuses of all genotypes, thereby suggesting that they might be artefactual. However, the control Parkes/NIH embryos were also cultured in FMP : 50% rat serum, and all 19 were normal after 24 hours in culture (Fig.28). This result suggested that the FMP : 50% rat serum was not generally toxic to embryos, but that it specifically affected the TGF-β1 knockout embryos.

3.4.6 Culture of TGF-β1 Knockout Embryos in FMP : 50% dCP plus TGF-β1.

Subsequent to the results obtained when TGF-β1 knockout embryos were cultured in FMP : 50% rat serum, an investigation was performed to determine whether the TGF-β in the rat serum was responsible for the observed increase in embryonic lethality. If embryonic abnormalities were not observed, these experiments would also investigate whether rTGF-β1 could rescue any of the phenotypic embryos.

To determine a tolerable concentration of rTGF-β1, ninety-two 8.5 dpc embryos from 15 Parkes/NIH matings were cultured in FMP : 50% dCP + variable concentrations of TGF-β1 (1000-25pg/ml) for 24 hours. Only the embryos cultured with 35pg or 25pg/ml rTGF-β1 survived the culture period. Therefore, the medium chosen to culture embryos from TGF-$\beta1^{+/-}$ x TGF-$\beta1^{+/-}$ matings was FMP : 50% dCP + 25pg/ml TGF-β1.

Fifty-one 8.5 dpc embryos from 6 TGF-$\beta1^{+/-}$ x TGF-$\beta1^{+/-}$ litters were cultured for 24 hours. During the culture period, 49 of the 51 embryos had died, regardless of genotype. Both the embryos and the yolk sacs exhibited defects (Fig.29, Table 11). To examine whether the 25pg/ml of rTGF-β1 was toxic to the TGF-β1 knockout embryos, a further 19 TGF-$\beta1^{+/-}$ intercross embryos were cultured in FMP : 50% dCP + 10pg/ml TGF-β1. 11 of these embryos died (Table 11). Genotype analysis identified 5 TGF-$\beta1^{-/-}$, 3 TGF-$\beta1^{+/-}$, and 3 TGF-$\beta1^{+/+}$ embryos, indicating that death was indiscriminate of genotype.

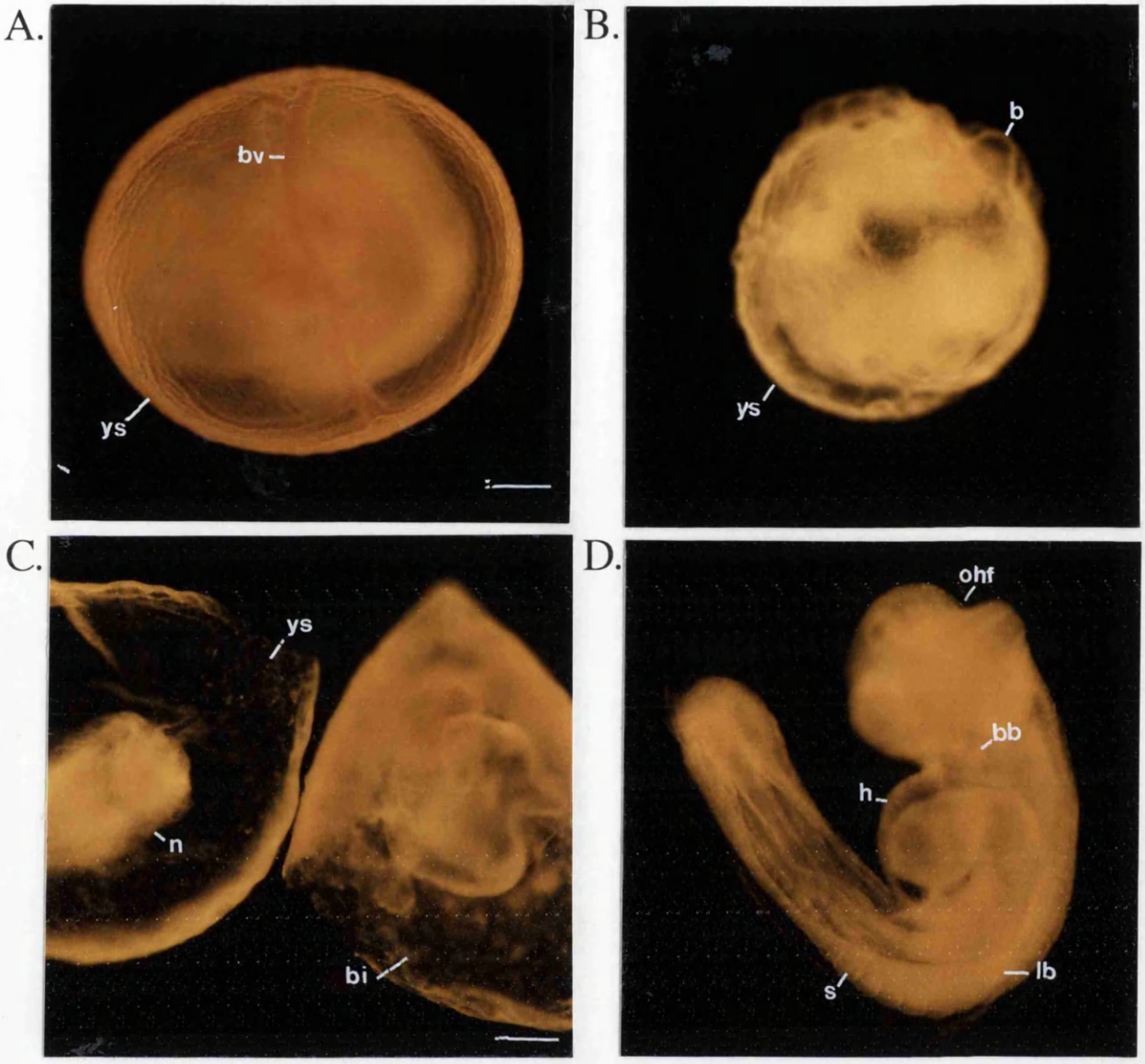

Figure 28 - Culture of TGF-β1 Knockout Embryos in FMP : 50% Rat Serum.

Attempts were made to rescue the abnormal phenotypes by culturing 8.5 dpc embryos in a medium containing TGF-β1. (A) Normal Parkes/NIH embryo after culture in FMP : 50% rat serum. (B) TGF-$\beta 1^{+/-}$ conceptus after culture. The yolk sac was bubbly although there were some vessels present, and the embryos development was delayed. (C) A severely necrosed TGF-$\beta 1^{-/-}$ embryo. The yolk sac vasculature developed to the blood island stage, then stopped. (D) TGF-$\beta 1^{+/+}$ embryo, delayed by approximately 0.5-0.75 days. Note the persistent open headfolds.

b - bubble; bb - branchial bar; bi - blood island; bv - blood vessel; h - heart; lb - limb bud; n - necrotic tissue; ohf - open headfolds; ov - optic vesicle; s - somite; ys - yolk sac.

Scale bars : (A,B) - 500μm ; (C,D) - 365μm.

Yolk Sac Phenotypes	Number of Yolk Sacs with Phenotype (Total = 75).		Embryo Phenotypes	Number of Embryos with Phenotype (Total = 75).
Normal	27 (36%)		Normal	29 (39%)
Necrosis	5 (7%)		Necrosis	28 (37%)
Bubbly	10 (13%)		Stunted Development	13 (17%)
"Skeletal"	3 (4%)		Hydrocephaly	1 (1%)
Disorganised vessels	7 (9%)		Anencephaly	5 (1%)
Reduced or no vessels	25 (33%)			
Anaemia	1 (1%)			

Table 10 - Culture of TGF-β1 Knockout Embryos in FMP : 50% Rat Serum.

8.5 dpc TGF-β1 knockout embryos were cultured in FMP : 50% rat serum for 24 hours to examine whether the TGF-β in the serum could rescue any of the TGF-$\beta 1^{+/-}$ or TGF-$\beta 1^{-/-}$ embryos destined to have an abnormal yolk sac. Instead of rescuing the embryos, the rat serum appeared to induce more abnormalities, regardless of genotype, with the embryos *per se* also affected. The defects and the number of samples which exhibited each phenotype are listed in the table above.

"skeletal" = transparent yolk sac, with no vessels, but thin processes attaching the yolk sac to the embryo. Looked like thin, long bones, therefore "skeletal".

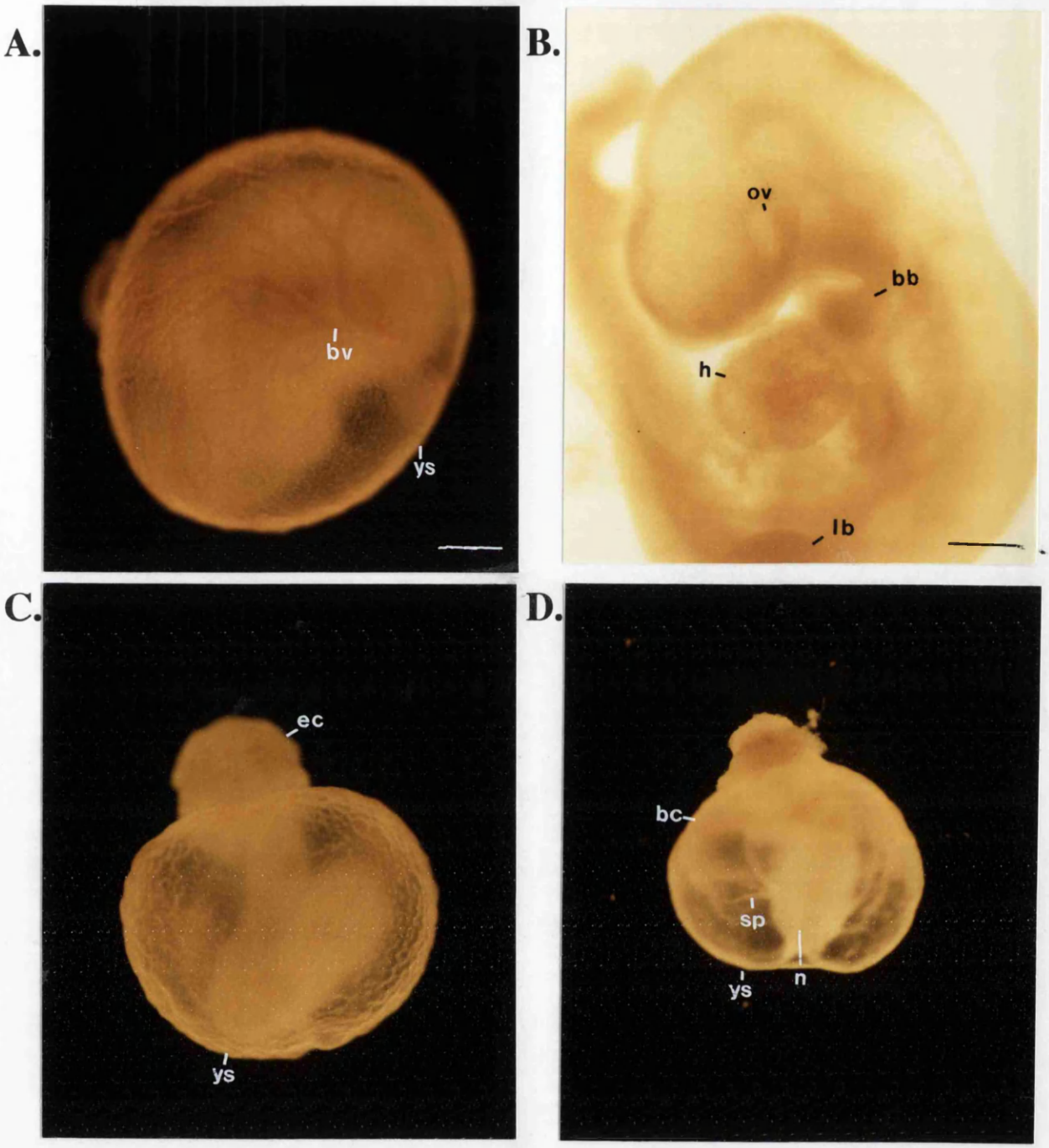

Figure 29 - Culture of TGF-β1 Knockout Embryos in FMP : 50% dCP + TGF-β1.

8.5 dpc embryos were cultured for 24 hours in FMP : 50% dCP + 25 pg/ml TGF-β1. (A,B) Normal Parkes/NIH embryo after culture. (C) TGF-$\beta1^{+/+}$ conceptus with abnormal vasculature. There are no proper vessels, but connections throughout the yolk sac. (D) A severely necrosed TGF-$\beta1^{+/-}$ embryo. This yolk sac exhibits the "skeletal" phenotype, where thin processes attach the yolk sac to the embryo.

bb - branchial bar; bc - blood corona; bv - blood vessel; ec - ectoplacental cone; h - heart; lb - limb bud; n - necrotic embryo; ov - optic vesicle; sp - "skeletal"processes; ys - yolk sac.

Scale bars : (A,C,D) - 500μm ; (B) - 365μm.

A.

Yolk Sac Phenotypes	Number of Yolk Sacs with Phenotype / Medium	
	25pg/ml TGF-β1	10pg/ml TGF-β1
Normal	0 (0%)	4 (21%)
Necrosis	2 (4%)	3 (15%)
Bubbly	16 (31%)	4 (21%)
"Skeletal"	19 (37%)	5 (26%)
Disorganised vessels	2 (4%)	3 (16%)
Reduced or no vessels	42 (82%)	4 (21%)
Anaemia	1 (2%)	2 (11%)

B.

Embryo Phenotypes	Number of Embryos with Phenotype / Medium	
	25pg/ml TGF-β1	10pg/ml TGF-β1
Normal	2 (4%)	8 (42%)
Necrosis	38 (74%)	11 (58%)
Stunted Development	9 (8%)	5 (26%)
Hydrocephaly	1 (2%)	1 (5%)
Anencephaly	4 (8%)	
Ballooned Allantois	1 (2%)	

Table 11 - Culture of TGF-β1 Knockout Embryos in FMP : 50% dCP + rTGF–β1.

Embryos were cultured in FMP : 50% dCP + rTGF-β1 to investigate whether the defects observed after culture in FMP : 50% rat serum were as a result of the TGF-β1 present in the serum. If the embryos did not develop abnormally, it would also examine if rTGF-β1 could rescue any of the phenotypic embryos. (A) Fifty-one 8.5 dpc embryos from TGF-$\beta1^{+/-}$ intercrosses were cultured for 24 hours in FMP : 50% dCP + 25pg/ml TGF-β1, before morphological examination. The embryos and yolk sacs exhibited a range of abnormalities, regardless of genotype. To examine whether 25pg/ml rTGF-b1 was toxic to the embryos, the concentration added to the medium was decreased to 10pg/ml (B). 19 embryos were cultured in this medium, and after culture they also exhibited defects. The range of phenotypes and their frequency after culture in both FMP : 50% dCP + 25pg/ml, and 10pg/ml rTGF-β1 is shown in the table above.

"skeletal" = transparent yolk sac, with no vessels, but thin processes attaching the yolk sac to the embryo. Looked like thin, long bones, therefore "skeletal".

These results show that the addition of very small amounts of TGF-β1 to the culture medium of 8.5 dpc TGF-β1 knockout embryos does not rescue any embryos, but rather that the exogenous TGF-β1 is detrimental to their development. The range of abnormal phenotypes was the same as that observed when the embryos were cultured in FMP : 50% rat serum, suggesting that TGF-β may be the causative factor.

3.4.7 An Investigation to Determine the Reason for Embryonic Lethality in Cultured TGF-β1 Knockout Embryos.

Studies were performed to investigate why embryos from TGF-$\beta1^{+/-}$ intercrosses died when cultured in FMP : 50% rat serum, or FMP : 50% dCP + 25pg/ml TGF-β1. There were two possibilities. Either gestation in a TGF-$\beta1^{+/-}$ mother conferred a higher sensitivity to TGF-β1 in the embryos than gestation in a TGF-$\beta1^{+/+}$ mother, or the genetic background of the embryos endowed a susceptibility to TGF-β1. If embryos gestated in a TGF-$\beta1^{+/-}$ mother did have increased TGF-β1 sensitivity, then TGF-$\beta1^{+/+}$ intercross embryos would develop normally in FMP : 50% rat serum and FMP : 50% dCP + 25pg/ml TGF-β1. However, if the genetic background of the TGF-β1 knockout mice was responsible, then the embryos from TGF-$\beta1^{+/+}$ intercrosses would also incur abnormalities, and die in culture. Therefore, sixty-one 8.5 dpc embryos from 6 TGF-$\beta1^{+/+}$ intercrosses were cultured in FMP : 50% rat serum, or FMP : 50% dCP + 25pg/ml TGF-β1. FMP : 50% dCP was the control medium. A further forty-one 8.5 dpc embryos from TGF-$\beta1^{+/-}$ intercrosses were cultured to directly compare with the TGF-$\beta1^{+/+}$ intercross embryos, alongside 15 control Parkes/NIH embryos. Embryos from each mating were divided between all three media to account for day-to-day variation.

As expected, the Parkes/NIH embryos developed normally, while the number and severity of the abnormalities observed in the TGF-$\beta1^{+/-}$ intercross embryos after culture in the different media was as before (Table 10, 11).

Of the TGF-$\beta1^{+/+}$ embryos, eighteen 8.5 dpc embryos were cultured in FMP : 50% dCP (Fig.30, Table 12). After 24 hours, all were phenotypically normal, except for 2 which were slightly necrotic. When FMP : 50% rat serum was used, 9 out of 17 embryos were abnormal after culture. However, as with the TGF-$\beta1^{+/-}$ intercross embryos, more abnormal embryos resulted from culture in FMP : 50% dCP + 25pg/ml TGF-β1. Only 4 of the 17 cultured embryos could be classified as normal, while the other 13 were defective. The phenotypes were similar to those observed in the embryos from TGF-$\beta1^{+/-}$ x TGF-$\beta1^{+/-}$ matings (Fig.30, Table 12).

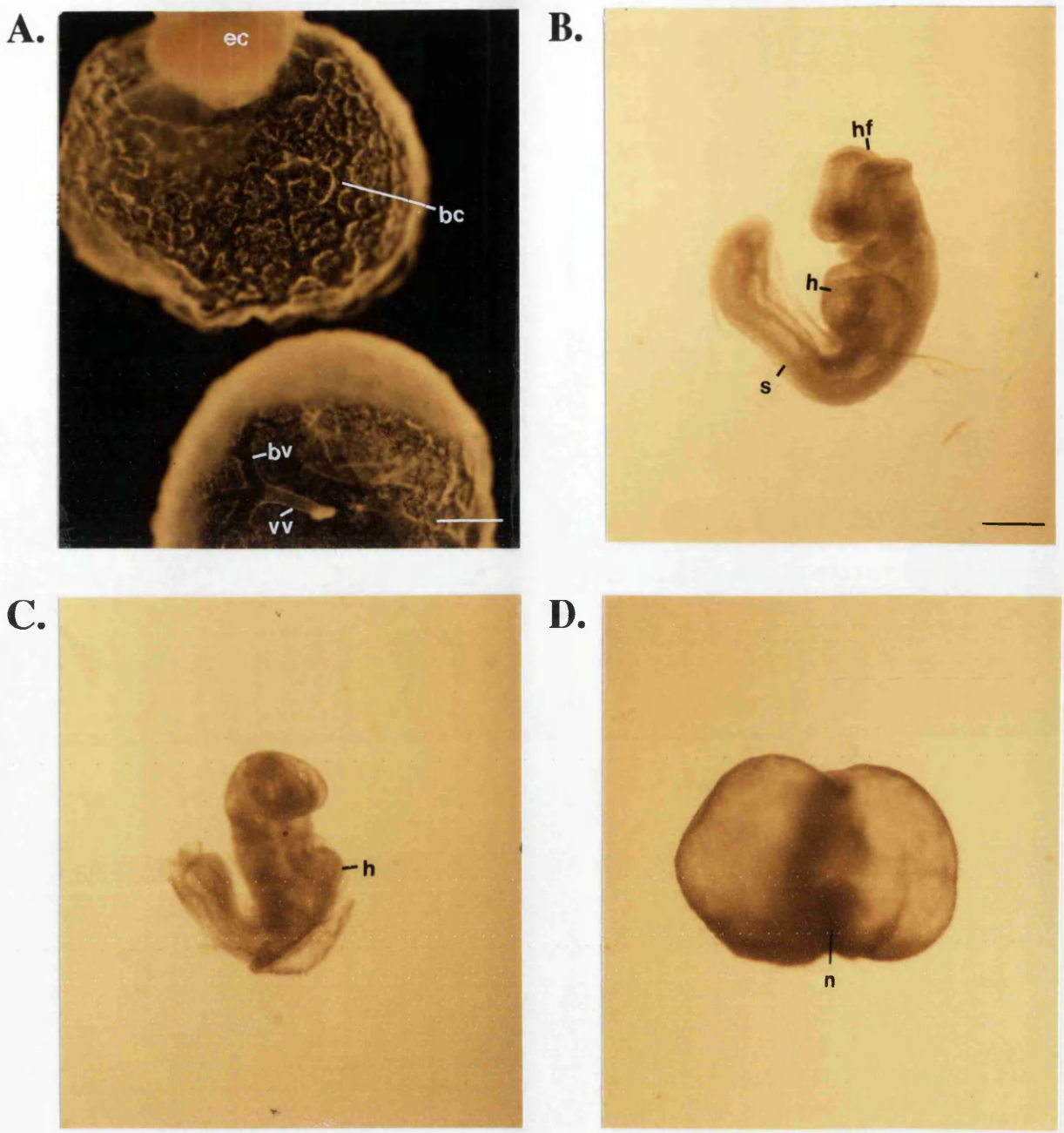

Figure 30 - Culture of Embryos from TGF-β1$^{+/+}$ Intercrosses.

8.5 dpc embryos from TGF-β1$^{+/+}$ x TGF-β1$^{+/+}$ matings were cultured for 24 hours in FMP : 50% rat serum, or FMP : 50% dCP + 25pg/ml TGF-β1. (A) Dissected yolk sac with completely disorganised vasculature, and (B) delayed embryo, equivalent to approximately 9 dpc, after culture in FMP : 50% rat serum. Note the persistent open headfolds. (C) Severely delayed embryo, approximately 8.5-8.75 dpc, and (D) extremely necrotic yolk sac and embryo after culture in FMP : 50% dCP + 25pg/ml TGF-β1.

bc - blood channel; bv - blood vessel; ec - ectoplacental cone; h - heart; hf - headfolds; n - necrotic embryo; s - somite; vv - vitelline vessel; ys - yolk sac.

Scale bars : (A) -300μm ; (B,C,D) - 450μm.

	FMP : 50% dCP (Total no. of embryos cultured=18)	FMP : 50% Rat Serum (Total no. of embryos cultured=17)	FMP : 50% dCP + 25 pg/ml TGF-β1. (Total no. of embryos cultured=17)
Normal	16 (89%)	8 (47%)	4 (24%)
Abnormal	2 (11%)	9 (53%)	13 (76%)

Table 12 - TGF-β1$^{+/+}$ x TGF-β1$^{+/+}$ Embryo Cultures.

8.5 dpc embryos from TGF-β1$^{+/+}$ intercrosses were cultured in FMP : 50% dCP, FMP : 50% rat serum, and FMP : 50% dCP + 25pg/ml TGF-β1 for 24 hours. This was performed to examine whether the abnormal phenotypes observed after culture in FMP : 50% rat serum, or FMP : 50% dCP + 25pg/ml TGF-β1 with the embryos from TGF-β1$^{+/-}$ intercrosses were as a result of gestation in a TGF-β1$^{+/-}$ mother, or their genetic background. Many of the TGF-β1$^{+/+}$ embryos cultured in this experiment were also abnormal after culture in FMP : 50% rat serum, or FMP : 50% dCP + 25pg/ml TGF-β1. The table above lists the ratio of normal versus abnormal embryos after culture in each medium. This indicated that the genetic background of the TGF-β1 knockout embryos conferred a sensitivity to culture in these media. FMP : 50% dCP was the control medium in which embryos from both crosses developed normally.

This data suggests that the increased number of defective phenotypes observed in TGF-β1 knockout embryos after culture in FMP : 50% rat serum, or FMP : 50% dCP + 25pg/ml TGF-β1 were as a result of their genetic background. The successful culture of the Parkes/NIH embryos in each of the media, demonstrated that the FMP : 50% rat serum, or FMP : 50% dCP + 25pg/ml TGF-β1 media were not inducing the defects themselves.

3.5 Analysis of TGF-β2, TGF-β3, and *Flk-1* in the TGF-β1 Knockout Embryos.

As mentioned in the Introduction, all three TGF-βs have a high degree of structural and functional similarity, especially TGF-β1 and TGF-β3. It was therefore postulated that TGF-β2, or TGF-β3 may assume the role of TGF-β1 in the transgenic TGF-β1 knockout embryos, thereby rescuing a number of the TGF-$\beta1^{+/-}$ and TGF-$\beta1^{-/-}$ embryos. An increase in the amount of TGF-β2 and/or TGF-β3 might be required to perform this additional role. The RNA levels of the two TGF-β isoforms were therefore analysed in the knockout embryos, using Northern analysis, and the Reverse Transcription Polymerase Chain Reaction (RT-PCR).

These methodologies were also used to examine the expression of *Flk-1*, a tyrosine kinase receptor for vascular endothelial cell growth factor (VEGF) which is specifically expressed in endothelial cells (Ferrara & Henzel, 1989). This was performed to examine whether the vascular abnormality observed in the TGF-β1 knockout yolk sacs was a result of defective endothelial cell differentiation.

When the RNA from individual half embryos of 8.5 or 9.5 dpc, and individual whole yolk sacs were analysed by Northern, TGF-β and *Flk-1* transcript levels were below the level of detection. Therefore, to obtain transcript levels that could be detected by Northern blotting, several RNA samples from genotypically and phenotypically identical embryos were pooled. Three 9.5 dpc TGF-$\beta1^{+/-}$ x TGF-$\beta1^{+/-}$ litters were used, resulting in 10 TGF-$\beta1^{+/+}$, 10 TGF-$\beta1^{+/-}$, and 8 TGF-$\beta1^{-/-}$ embryos. Half the embryo was used for genotyping, and the other half for RNA extraction. Three half embryo or 3 yolk sac RNA samples were then combined and subjected to Northern blot analysis, as described in Materials and Methods.

Bands were observable when the blot was probed with TGF-β3. The 3.5kb TGF-β3 mRNA transcript was identified, and found in both embryo and yolk sac samples of all genotypes (data not shown). It was observed that neither the yolk sac or embryo of

any genotype or phenotype had increased levels of TGF-β3, suggesting that this isoform was not upregulated in response to a decrease in TGF-β1. However, the phenotypic TGF-$\beta1^{-/-}$ samples exhibited slightly reduced TGF-β3 transcript levels in comparison with the other samples. The level of RNA present in each sample was therefore examined using 7s RNA.

7s RNA is normally ubiquitous and abundant in all cells. When the blot was probed with 7s RNA, it was noted that the phenotypic TGF-$\beta1^{-/-}$ embryos and yolk sacs contained considerably less 7s RNA than the other genotypes (Fig.31), thereby explaining the reduction in TGF-β3 transcript levels also found in these samples. The reduction in the level of 7s RNA in the phenotypic TGF-$\beta1^{-/-}$ samples was observed in a further 2 Northern blots containing individual half embryo, and yolk sac RNA. This may have been due to an increase in cell death within these embryos, or the conceptuses could have been of a smaller size.

Due to the fact that Northern methodology was unable to detect TGF-β2 or *Flk-1* transcripts, an attempt was made using RT-PCR. This method enables the detection of a transcript that is present in very small amounts. Primers were designed specific for the transcript of interest (Table 4). RT-PCR was also performed for TGF-β3 to consolidate the Northern analysis, and for GAPDH. GAPDH transcripts are present in equal amounts in most cell types, and therefore can be used as a control.

To detect the expression of the different mRNA transcripts in embryos or yolk sacs, 9 TGF-$\beta1^{+/+}$, 27 TGF-$\beta1^{+/-}$, and 14 TGF-$\beta1^{-/-}$ conceptuses were obtained from 6 TGF-$\beta1^{+/-}$ x TGF-$\beta1^{+/-}$ matings. These samples were treated like those for the Northern analysis to obtain pooled RNA samples, before reverse-transcribing and using in the RT-PCR, as described in Materials and Methods. The RT-PCRs were performed twice.

When the RT-PCR products were analysed after electrophoresis in an agarose gel, the results indicated that there was no difference in TGF-β2 mRNA levels between the TGF-β1 knockout embryos or yolk sacs of different genotypes or phenotypes (Fig.32). This suggested that TGF-β2 was not up-regulated in response to a decrease, or the absence of TGF-β1 in the TGF-β1 knockouts. The RT-PCRs for TGF-β3 and for GAPDH both failed.

Interestingly, the results for *Flk-1* strongly suggested that there was a difference in *Flk-1* expression between the samples (Fig 33). However, instead of a difference in the phenotypic yolk sacs, as was expected, the expression levels were found to differ

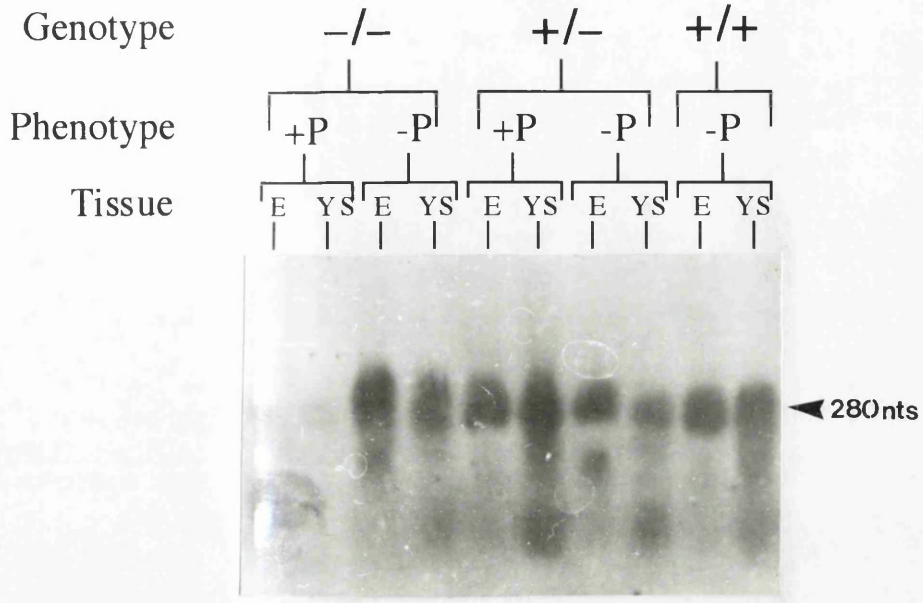

Figure 31 - Detection of 7s RNA.

To be able to detect TGF-β2, TGF-β3, or *Flk-1* transcripts in the TGF-β1 knockout embryos, or yolk sacs by Northern analysis or PCR, the RNA from 3 1/2 embryos, or 3 yolk sacs had to be combined. Combined RNA samples were obtained from conceptuses of like genotype and phenotype. The photograph above shows an autoradiograph of a Northern blot containing the combined RNA samples probed with 7s RNA. This was performed to ensure that the RNA was present, and could be detected. Note the decreased levels of 7s RNA in the phenotypic TGF-$\beta 1^{-/-}$ embryo and yolk sac samples.

+P - Abnormal yolk sac phenotype.
-P - Normal yolk sac phenotype.
E - 1/2 Embryo samples.
YS-Yolk Sac Samples.

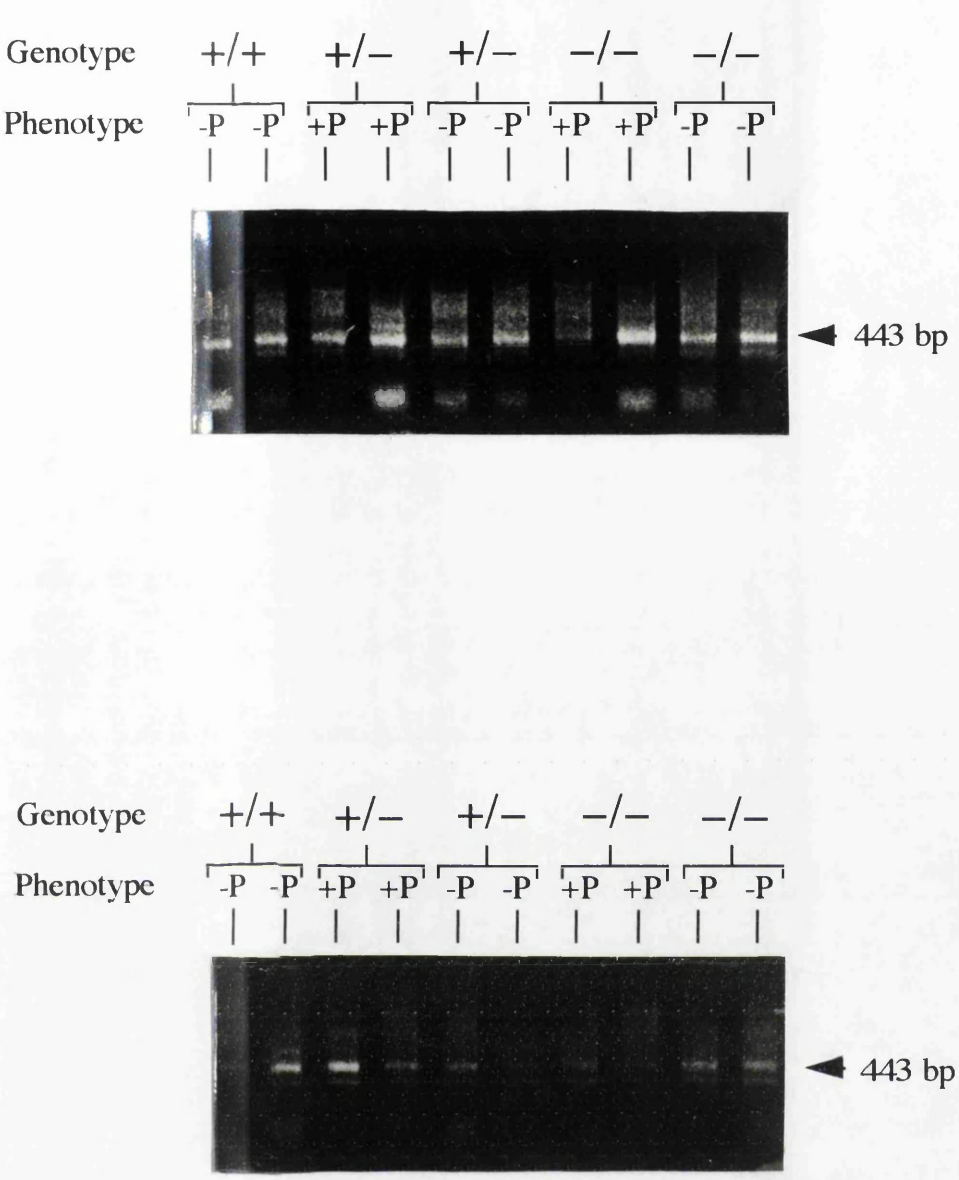

Figure 32 - TGF-β2 RT-PCR with Embryo and Yolk Sac RNA.

The combined RNA from 9.5 dpc TGF-β1 knockout embryos, or yolk sacs was reverse-transcribed before using in the PCR. The photographs above show the results of one such PCR for TGF-β2. There were two samples of each genotype plus phenotype for both embryos (A) and yolk sacs (B). The transcript size was 443bp. The diffuse bands at the bottom of the photos are excess primers.

+P - Abnormal yolk sac phenotype.

-P - Normal yolk sac phenotype.

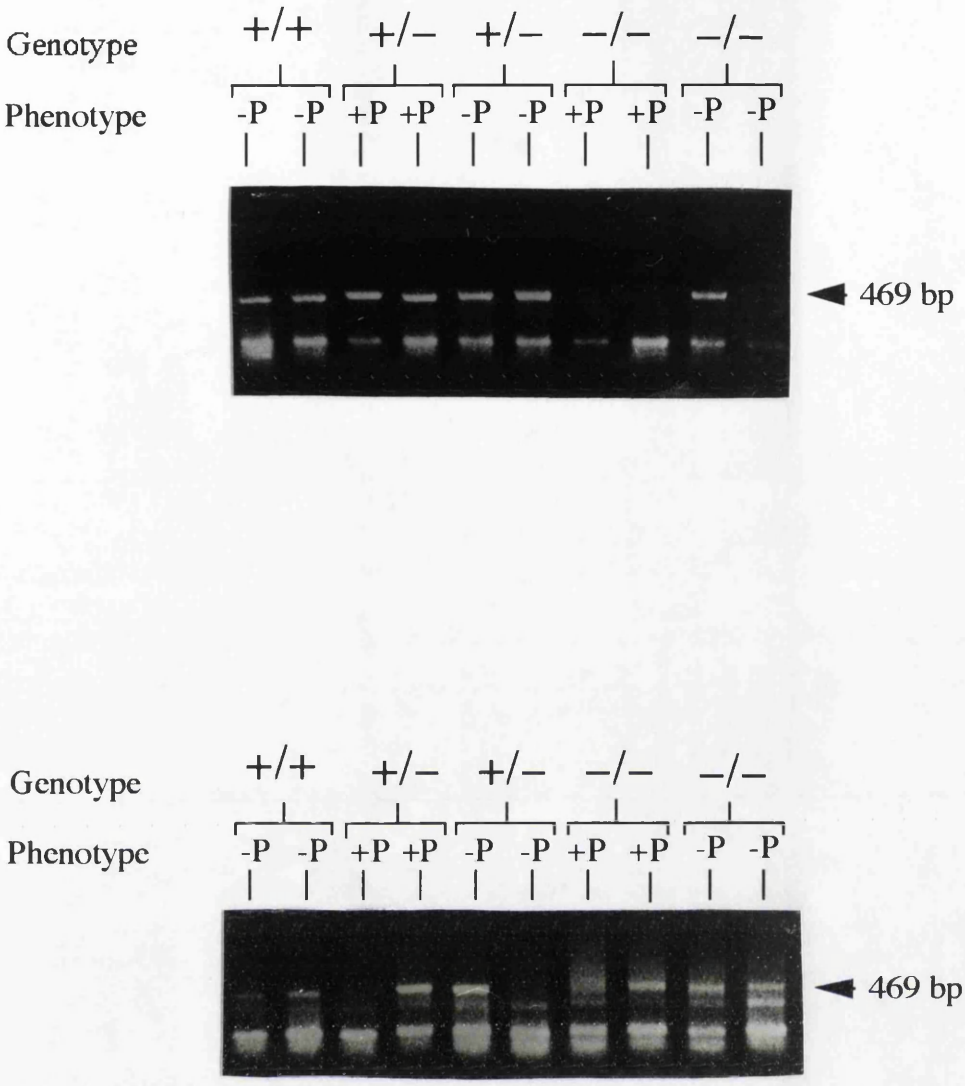

Figure 33 - *Flk-1* RT-PCR with Embryo and Yolk Sac RNA.

The combined RNA from 9.5 dpc TGF-β1 knockout embryos, or yolk sacs was reverse-transcribed before using in the PCR. The photographs above show the results of one such PCR for the endothelial cell receptor tyrosine kinase, *Flk-1*. There were two RNA samples of each genotype plus phenotype for both embryos (A) and yolk sacs (B). The transcript size was 469bp. The intense diffuse bands at the bottom of the photographs are probably primer dimers.

+P - Abnormal yolk sac phenotype.

-P - Normal yolk sac phenotype.

in the embryos. The RT-PCR products showed a reduced *Flk-1* expression in the TGF-β1$^{-/-}$ embryos that had been contained within a phenotypic yolk sac. All other embryos, had approximately equal amounts, regardless of their genotype, or the phenotype of their yolk sac. Some of the yolk sac samples *per se* also showed reduced *Flk-1* transcript levels, but these were not consistent with genotype or phenotype, and were therefore ambiguous (Fig.33)

The major quandary with the *Flk-1* results is the difference in the TGF-β1$^{-/-}$ embryos *per se*, which do not have a phenotype, but no consistent difference in the phenotypic yolk sacs. This is also in disagreement with earlier results from Dr.M.Dickson, who found a difference between the expression of *Flk-1* in the yolk sacs of different genotypes by whole-mount *in situ* hybridisation (Dickson *et al*, 1995). However, as for the RT-PCR, Dr.M.Dicksons experiments were performed only twice, and would need to be repeated to attain significant numbers and results. One further difference between Dr.M.Dicksons results and the RT-PCR results found in this study was the age of the conceptuses. Dr.M.Dickson examined 8-8.5 dpc embryos, whereas the RT-PCR utilised 9.5 dpc embryos.

3.6 Maternal or Paternal Imprinting of TGF-β1.

Genomic imprinting is the specific inactivation of the maternal or paternal copy of a gene. Imprinted genes identified so far include Igf2, Igf2r, H19, and ins1 and ins2 (DeChiara *et al*, 1991; Bartolome *et al*, 1991; Giddings *et al*, 1994). A gene may be imprinted in all cells of the body, or only in a specific tissue, as is the case with insulin 1 and 2, which are imprinted only in the yolk sac (Giddings *et al*, 1994).

A number of observations on the TGF-β1 knockout mice suggested that TGF-β1 might also be imprinted. When a female TGF-β1$^{+/-}$ was crossed with a male TGF-β1$^{+/+}$, the ratio of +/- : +/+ offspring was 0.8 : 1. However, the offspring of the reciprocal cross were in the ratio of 1 : 1 (Dickson *et al*, 1995). This suggested that the paternal copy of the TGF-β1 gene was imprinted or there was a maternal biological effect in TGF-β1 females. The fact that the abnormal phenotypes observed in the TGF-β1$^{+/-}$ and TGF-β1$^{-/-}$ embryos were restricted to the yolk sac, suggested that, like ins1 and ins2, TGF-β1 may be imprinted specifically in the yolk sac. To investigate these hypotheses, reciprocal male-female crosses were used to determine whether the gene was imprinted on the maternal or paternal allele. Seven 9.5 dpc embryos were removed from a female TGF-β1$^{+/+}$ x male TGF-β1$^{+/-}$ mating, and 8 from a male TGF-β1$^{+/+}$ x female TGF-β1$^{+/-}$ mating. Half of each embryo was used

for genotyping, whilst RNA was extracted from the other half embryo and from individual yolk sacs. Each embryo and yolk sac RNA sample was then subjected to Northern blot analysis, as described in Materials and Methods. The 2.7kb TGF-β1 transcript was detected in embryo and yolk sac samples of both crosses (Fig 34) suggesting that TGF-β1 is not imprinted, either throughout the conceptus, or specifically in the yolk sac. However, Northern analysis could not exclude the possibility of cell-type specific imprinting in a small population of cells.

The reason for different +/- : +/+ ratios in reciprocal crosses must therefore be due to a biological maternal effect. This is most likely to be a reduction in circulating levels of TGF-β1 in the TGF-$\beta1^{+/-}$ mother in comparison to the TGF-$\beta1^{+/+}$ mother. This could be examined by analysing TGF-β1 levels in the serum of these animals by CCL64 assay.

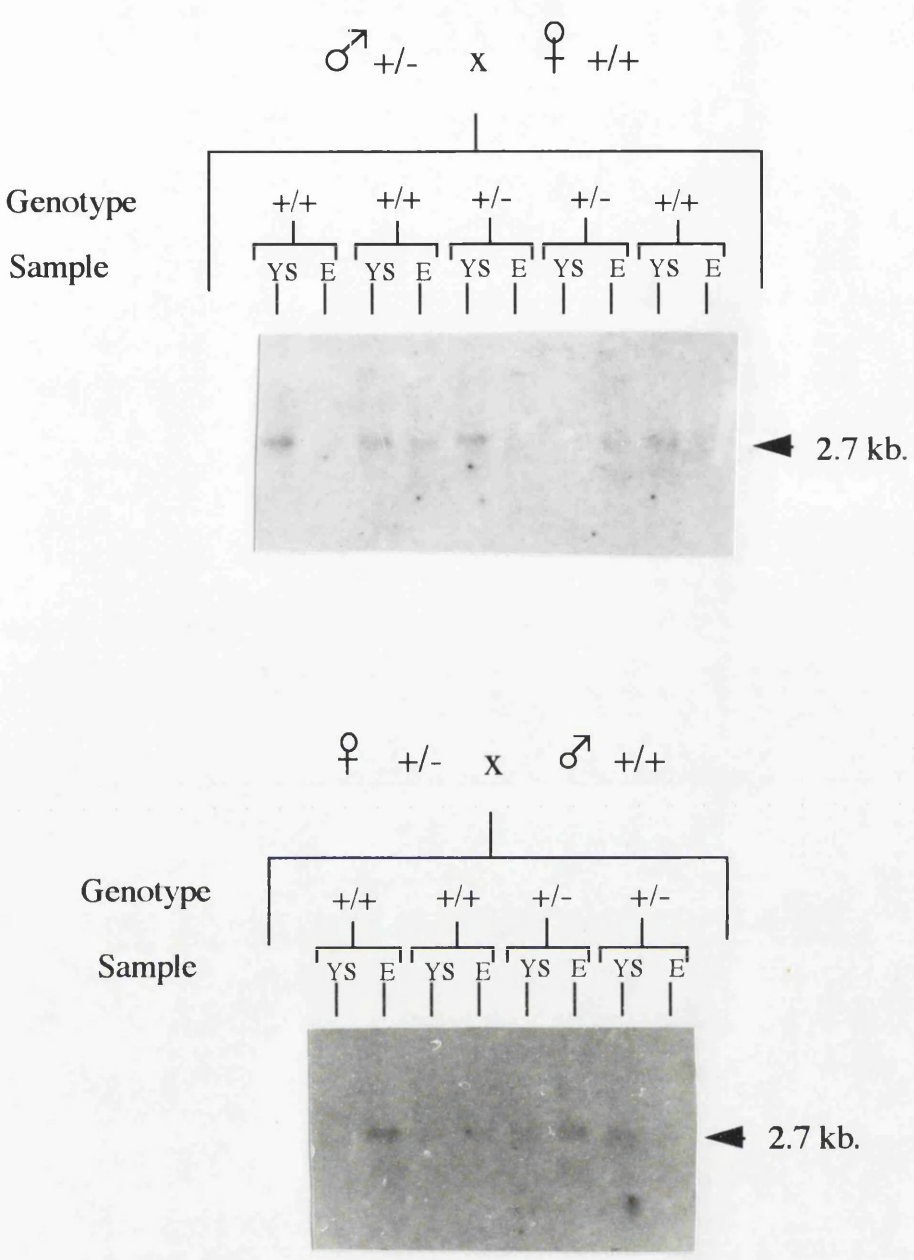

Figure 34 - TGF-β1 Imprinting.

The RNA from individual 1/2 embryos (E) or their yolk sacs (YS) was probed with a TGF-β1 cDNA to determine if this gene was imprinted. Conceptuses from reciprocal TGF-$\beta1^{+/-}$ x TGF-$\beta1^{+/+}$ matings were used. If paternal imprinting was present, one would expect to see loss of expression of the TGF-β1 gene in heterozygous offspring when the father was the source of the wild-type allele (ie when mother was TGF-$\beta1^{+/-}$) (B). This is clearly not the case, as the 2.7kb TGF-β1 transcript was detected in both yolk sac and embryos of heterozygous embryos, regardless of the direction of the cross.

Chapter 4

DISCUSSION

4.1 Introduction.

This thesis presents data obtained from *in vivo* and *in vitro* analyses of TGF-β1 knockout embryos (Shull *et al*, 1992; Kulkarni *et al*, 1993). Previous studies demonstrated the expression pattern of TGF-β1 mRNA and protein during embryogenesis, and also suggested possible actions of this growth factor *in vivo* (Heine *et al*, 1987; Lehnert & Akhurst, 1988; Akhurst *et al*, 1990; Roberts & Sporn, 1990, for review). However, its true importance *in vivo* could not be elucidated until it was possible to remove the protein, and examine the consequences of this action. This was recently achieved by two separate groups using gene targeting by homologous recombination to selectively knockout the TGF-β1 gene, and create transgenic null mice (Shull *et al*, 1992; Kulkarni *et al*, 1993). Surprisingly, mice of all genotypes were born, suggesting that TGF-β1 was not as important for foetal development as had been thought. However, on closer examination it was apparent that a percentage of TGF-$\beta1^{+/-}$ and TGF-$\beta1^{-/-}$ embryos died *in utero*. The work accomplished in this project set out to determine the cause of this prenatal lethality, and found that abnormal yolk sac development resulted in the death of approximately 50% of TGF-$\beta1^{-/-}$, and 25% of TGF-$\beta1^{+/-}$ embryos.

A second objective of the work was to investigate whether the surviving embryos were rescued by maternal TGF-β1. As the yolk sac phenotype was first observed at 9.5 dpc, 8.5 dpc embryos from TGF-$\beta1^{+/-}$ x TGF-$\beta1^{+/-}$ matings were cultured in a novel post-implantation embryo culture system depleted of TGF-β1. It was expected that, if maternal TGF-β1 was rescuing the abnormal embryos at this stage, then there would be an increase in embryos manifesting the abnormal phenotype after *in vitro* culture. No increase in abnormal embryos was observed after 24 hours. This did not

exclude the possibility that maternal TGF-β1 rescues embryos *in vivo*, but suggests that if it does occur, then it does so before 8.5 dpc.

4.2 Post-Implantation Embryo Culture in TGF-β1-Depleted Medium.

One of the main methods to be employed in this project was post-implantation embryo culture. As no-one in the research group had previously used *in vitro* post-implantation embryo culture, existing culture media and techniques were compared. In addition, this work required that the medium be depleted of TGF-β. Therefore, existing and novel culture media were analysed for TGF-β content.

In the past, the first successful post-implantation embryo culture experiments were performed with 100% rat serum, a medium which is still in use today (Steele & New, 1974; New *et al*, 1976; Pratten *et al*, 1988; Walsh *et al*, 1993). However, it has since been discovered that although a percentage of the medium has to be rat serum, it is possible to use a combination of rat serum, with a chemically-defined, commercial medium, or with saline (Tam & Beddinton, 1987; Savatier *et al*, 1990; Shepard *et al*, 1993; Guest *et al*, 1993; Smoak *et al*, 1993; Augustine *et al*, 1993; Akawaza *et al*, 1994). As yet, a medium that can support early post-implantation embryonic development without containing serum, or some other undefined biological component has not been found (Cockroft, 1979; Priscott *et al*, 1983).

In this analysis, rat serum or plasma was combined with commercial media. Normal embryonic development occurred when 50% or more of the medium was defined. This reduced the amount of unknown factors, and saved on the precious commodity of rat serum or plasma.

4.2.1 The Use of Dulbeccos' Modified Eagles Medium.

DMEM : 50% rat serum has been successfully employed by other groups (Tam & Beddington, 1987; Savatier *et al*, 1990; Kesby, 1992; Shepard *et al*, 1993). These studies used mouse embryos of 8-10 dpc, or rat embryos of the equivalent stages. The culture periods lasted for 20-48 hours, with one or two changes of the medium. The medium was also gassed intermittently to maintain a pH of 7.1-7.3, and the oxygen concentration was adjusted to be optimal for the age of the embryo. 5% O_2, 5% CO_2, and 90% N_2 was used for early somite embryos, 20% O_2, 5% CO_2, and 75% N_2 for 10-15 somite embryos, 40% O_2, 5% CO_2, and 55% N_2 for 20-30 somite embryos, and

95% O_2, 5% CO_2 for 30 somite embryos and above (New *et al*, 1976; Morriss & New, 1979; Cockroft, 1990). None of these studies reported any abnormal embryos resulting from the *in vitro* culture, implying that the use of DMEM : 50% rat serum was ideal for standard practice. However, the results with DMEM : 50% rat serum in this project were relatively poor.

Cultures of 7.5 dpc embryos were quite successful, although there were a few necrotic embryos, and development was slightly delayed in comparison to embryos developing *in vivo*. However, when 8.5 dpc embryos were cultured in DMEM : 50% rat serum, as well as necrosis, a number of embryos also exhibited structural abnormalities. This discrepancy between the 7.5 and 8.5 dpc embryo cultures suggested that DMEM : 50% rat serum was only suitable for culturing younger embryos. The older embryos may have been utilising more of the nutrients in the medium. Embryos do exhaust the medium of certain factors during development, and so changing the medium of the 8.5 dpc embryos during the course of the culture period would have revealed if depletion of nutrients was resulting in the necrosis and developmental defects (Priscott *et al*, 1983; Pratten *et al*, 1988).

In contrast, other groups have successfully cultured 8-10 dpc embryos in DMEM : 50% rat serum. The deleterious effect seems to be specific to this study, although it must be pointed out that not all of the embryos were abnormal. As the other studies were not analysing culture methods, it is possible that they did not report any abnormal development. Nevertheless, there were two major differences between the cultures performed in this project, and in the other studies. One is the strain of mice used, and the other is the gas equilibration of the media.

In this study, outbred NIH/Parkes or inbred NIH mice were used, whereas others use outbred PO (Pathology, Oxford) mice, or Wistar rats (Tam & Beddington, 1987; Savatier *et al*, 1990; Kesby, 1992). Culture of the embryos may differ due to strain variation in the level of nutrients required, the speed at which the nutrients are utilised during embryogenesis, or differences in sensitivity to certain factors present in the medium. Data in support of strain variation was obtained in the current study by the culture of wild-type TGF-β1 knockout embryos in a number of different media. As described previously, 8.5 dpc embryos from Parkes/NIH or NIH matings developed normally in either FMP : 50% dCP or FMP : 50% rat serum. However, only FMP : 50% dCP could support the normal development of 8.5 dpc embryos from TGF-$\beta1^{+/+}$ x TGF-$\beta1^{+/+}$ matings. Culture in FMP : 50% rat serum for 24 hours resulted in abnormal embryonic development. Therefore, there appeared to be a difference between the genetic background of the Parkes/NIH and NIH embryos in comparison

to the TGF-β1 embryos which determined their suitability to culture in FMP : 50% rat serum.

The other major difference between this study and others involves the equilibration of the medium. It is suggested that for culturing 8.5 dpc embryos, the medium is pre-equilbrated with 5% O_2, 5% CO_2, and 90% N_2 (New *et al*, 1976; Morriss & New, 1979; Cockroft, 1990). Those studies found that if early somite embryos were exposed to more than 10% O_2, there was a concommitant increase in the number, and the severity of defects in the cultured embryos. The neural tube was most commonly affected, specifically in the brain area, with defects ranging from failure of the anterior neuropore to close, to wide-open neural folds the length of the head (New *et al*, 1976, Morriss & New, 1979). However, very early on in this project, it became apparent that when 5% O_2, 5% CO_2, and 90% N_2 was used, many of the media became acidic very rapidly during the culture period with the result that the embryos died. Therefore, an attempt was made to culture the embryos in medium equilibrated with 20% O_2, 5% CO_2, and 75% N_2. The cultured embryos showed no obvious increase in defects as a result of this change, regardless of the medium they were cultured in, and the pH was maintained at 7.1-7.4 for at least 14-16 hours, before the medium was gassed again. In contrast, other groups do use 5% O_2, 5% CO_2, and 90% N_2 after equilibrating the medium overnight in 5% CO_2 in air (Tam & Beddington, 1987; Savatier *et al*, 1990), and do not obtain pH problems.

Apart from pre-equilibrating with 5% CO_2 in air, the only other difference between the current study and others was the volume of medium, and the size of the culture bottles used. 1ml of medium in a 20ml Universal was used in this study to culture 1 embryo, whereas 3 or 6mls of media were used to culture multiple embryos in 30 or 60ml bottles in the other groups (Cockroft, 1979; Morriss & New, 1979; Tam & Beddington, 1987; Savatier *et al*, 1990). It is possible that the increase in medium, and the larger volume of the 30 or 60ml bottle would retain more of the gas mixture, thereby enabling the pH to be maintained for a longer period of time. This could be investigated by culturing embryos in varying volumes of DMEM : 50% rat serum in a variety of different sized bottles, and gassing the medium with 5% O_2, 5% CO_2, and 90% N_2.

4.2.2 The Use of Foetal Mouse Palate Medium.

Previously, FMP, a serum-free culture medium, had been used to culture isolated foetal mouse palates (Shiota *et al*, 1990). As this was successful without the need for the addition of serum, in this study, an attempt was made to use FMP as a fully defined medium for whole embryo culture. Both 7.5 and 8.5 dpc embryos were cultured in this media. The results indicated that although FMP alone could not support embryonic development, it was extremely effective when combined with rat serum, especially when used with 8.5 dpc embryos. 50% serum was most efficient, but it was possible to decrease the organic component down to as little as 10%, and still maintain relatively good development. In FMP : 10% rat serum, the most common problem was necrosis, rather than embryonic abnormalities. However, because the necrosis was not severe, if the culture time period was reduced from the 24 hour period used here, to perhaps 12 hours, then it would be unlikely that necrosis would occur at all. It may also be possible to culture older embryos, approximately 11 dpc and on, in FMP without supplementation (J.McCluskey, pers. comm.). FMP appears to be an extremely useful medium for post-implantation embryo culture.

4.2.3 The Use of Rat Plasma in Embryo Culture.

Although rat serum is commonly used, plasma has been utilised very rarely as an embryo culture medium. Both of these fluids are obtained from blood, but their collection and their components differ. Serum is an amber-coloured liquid which is a by-product of blood coagulation, whereas plasma is the natural liquid component of blood which contains all the blood cells, fat globules, coagulating factors, etc. To prepare serum, the blood is allowed to clot, whereas it is imperative that clotting does not occur if plasma is required.

The first plasma samples that were used for culturing embryos were obtained using calcium chelating anticoagulants. However, once in the culture medium these agents upset the physiological concentration of free calcium ions in the embryo (Ellington, 1983; Smedley & Stanisstreet, 1985). Therefore heparin, a naturally occurring anticoagulant, is now used to prepare plasma for use in embryo culture (Kesby, 1992). When plasma was first used for culture, pre-somite and early somite embryos with intact yolk sacs and Reicherts membranes were cultured in 100% plasma in watchglasses exposed to air, but only very limited development was observed (Nicholas & Rudnick, 1934). Plasma was only used again when more advanced culture methods had been devised, and rather than simply using watchglasses exposed to air, roller bottles were utilised, with intermittent gassing of the medium. However,

once again, early somite embryos were cultured in 100% heparin-plasma (Sanyal & Wiebe, 1979), with the result that embryonic development was significantly retarded. Kesby (1992) suggested that the resulting abnormalities were due to the large concentrations of heparin used by Sanyal & Wiebe (1979) to extract the plasma. The minimum amount required for successful plasma extraction is recorded as approximately 20 IU/ml (Young & Bermes, 1986), whereas Sanyal & Wiebe had used 100 IU/ml. Therefore, Kesby (1992) examined if the observed abnormal embryonic development was a result of the heparin concentration by culturing embryos in DMEM : 50% plasma, using plasma samples that had been extracted with 10-80 IU heparin per ml blood. The results indicated that embryos were affected by heparin at a concentration as low as 5 IU heparin per ml media. Therefore, most investigators decide not to use plasma for *in vitro* post-implantation embryo culture. However, there were several advantages for the use of plasma in comparison to serum in this project, and so another investigation into the use of plasma as a post-implantation embryo culture medium was performed here.

4.2.3.1 Rat Plasma in comparison to Serum for Embryo Culture.

As mentioned previously, one of the most important factors of the culture medium required for this project was that it lacked TGF-β. Plasma was considered for using in the culture medium, as it was already known that if the platelets were removed there would probably be no, or undetectable amounts (<25pg/ml) of TGF-β remaining (Danielpour *et al*, 1989). Platelets are one of the most abundant sources of TGF-β in the body, and once they were removed, it was thought that the TGF-β would also be removed (Van den Eijnden-van Raaij *et al*, 1988). Therefore, in an attempt to deplete the TGF-β from the plasma, the plasma was subjected to a further centrifugation process, thereby removing the platelets.

Analysis of the TGF-β levels in this centrifuged plasma (dCP) indicated that there were very, very low levels present (<25pg/ml). When the serum was analysed for TGF-β, it was found to contain much greater amounts (17ng/ml). This difference between plasma and serum TGF-β concentration could be as a result of a number of factors. Firstly, the method of preparation. With the formation of a clot for serum, it is possible that the platelets rupture releasing their contents into the serum. As the clot is prevented from forming by anticoagulant agents in the preparation of plasma, the platelets remain intact. Second, the extra centrifugation steps that the plasma sustains, but not the serum, may remove the TGF-β within the platelets as described above. Or thirdly, the anticoagulation agent, heparin, may bind to and effectively remove the activity of TGF-β. Latent TGF-β1 is known to bind to heparin-Sepharose, suggesting

the presence of a specific heparin binding site in the LAP (Wakefield *et al*, 1989). However, regardless of the way in which TGF-β is depleted from the plasma, it is depleted to such an extent that it is undetectable by a highly sensitive CCL64 assay.

The CCL64 assay uses mink lung epithelial cells, cells which are growth inhibited by TGF-β *in vitro* (Danielpour *et al*, 1989). However, in almost every assay performed, there was a slight growth stimulatory effect at low concentrations of the standard rTGF-β1. Although this stimulatory effect at low levels has not been observed in most other studies, including CCL64 assays (Baird & Durkin, 1986; Frater-Schroder *et al*, 1986; Longstreet *et al*, 1992; Zauli *et al*, 1993; Slager *et al*, 1993; Raynal & Lawrence, 1995), there are a few other investigations where it has been detected. These include the growth stimulatory effect of 25pg/ml TGF-β on porcine aortic smooth muscle cells in culture, and the increase in endothelial cell proliferation peripheral to the site of TGF-β1 application in chorio-allantoic membranes (Yang & Moses, 1990; Hwang *et al*, 1992).

It was this stimulation at low rTGF-β concentrations that suggested that there was no, or very, very low levels (<10pg/ml) of TGF-β1 present in plasma. The cells were not inhibited in the presence of dCP, and there was no significant increase in tritiated thymidine (^{3}H) incorporation when the anti-TGF-β1 antibody was applied. However, the fact that there was no statistically significant decrease in ^{3}H incorporation when the antibody removed the protein also suggested that growth stimulatory levels of TGF-β1 were not present either. If stimulatory levels of TGF-β were present, then the proliferation of the cells should have significantly decreased with the removal of the protein.

Detection of the low level of TGF-β1 that stimulated the cells also suggested that in the current study, the CCL64 assay was particularly sensitive. Other studies fail to detect a difference between concentrations of TGF-β1 below approximately 50-75 pg/ml (Longstreet *et al*, 1992; Zauli *et al*, 1993; Slager *et al*, 1993). The reason for the increased sensitivity of the cells to TGF-β in this study in comparison to others could be due to the quality of the cells in culture. Certainly, mink lung epithelial cells do lose their inhibition to TGF-β if they become confluent *in vitro*. It is also possible that other groups do not observe a stimulatory effect due to the quality of their recombinant TGF-β1.

The fact that TGF-β1 was depleted from the plasma raises the possibility that other growth factors may also have been removed by the processing of the plasma. It would

therefore be interesting to analyse the dCP for proteins such as platelet-derived growth factor, or epidermal growth factor.

The observation that TGF-β was absent from dCP made the plasma ideal for embryo culture use in this project. Therefore, attempts were made with the dCP to see whether it would support embryonic development, or would induce abnormalities as had been observed in the other studies (Nicholas & Rudnick, 1934; Sanyal & Weibe, 1979; Kesby, 1992).

4.2.3.2 DMEM or FMP plus Rat Plasma.

To analyse the effectiveness of dCP for whole post-implantation embryo culture, dCP was combined with DMEM or FMP and used to culture 7.5 and 8.5 dpc embryos for 24 hours. As described in 4.2.2, when embryos were cultured in DMEM : 50% rat serum, there was a difference in the number of abnormalities depending on the age of the embryo. However, when DMEM was combined with rat plasma, defective phenotypes were observed in embryos cultured from 7.5-8.5 dpc, or from 8.5-9.5 dpc. There was also an increase in the number of abnormal, and necrotic embryos, especially in the 7.5 dpc cultures. These results therefore appeared to suggested that the plasma was harmful to the embryos. However, when the dCP was combined with FMP, the 7.5 and 8.5 dpc embryos developed well, with only a few abnormal and/or necrotic embryos observed after culture. This observation thus refuted the hypothesis that the defective phenotypes in DMEM : 50% dCP were as a result of the plasma. Therefore, it had to be an effect of the plasma in combination with the DMEM.

From the observed increase in necrosis when dCP was used rather than rat serum, the initial implication was that the DMEM : 50% dCP was nutritionally deficient. If this were the case, media with a decreased amount of plasma would be even more nutritionally insufficient. This was found to be true for embryos cultured in FMP : 10% dCP. These embryos did not develop to the same stage as their counterparts in FMP : 50% dCP, and also developed more abnormalities, especially for the 7.5 dpc embryos. However, 7.5 or 8.5 dpc embryos cultured in DMEM : 10% dCP developed further, resulting in an approximately 50% decrease in the number of abnormal or necrotic embryos, in comparison to the embryos cultured in DMEM : 50% dCP. This statistically significant difference strongly suggested that DMEM : 50% dCP was not nutritionally deficient, but rather that it was toxic. With an increase in plasma, there was an increase in toxicity. It is possible that there is a factor(s) present in DMEM, which is not found in FMP, which reacts with a factor(s) present in the plasma. This

might result in the formation of toxic metabolites, which impinge on, and disrupt embryonic development.

These observations also agreed with those of Kesby (1992). In his study, the heparin-plasma was combined with DMEM, with the result that the embryos grew abnormally. Kesby also combined heparin with DMEM : 50% rat serum, and observed abnormal development, therefore suggesting that the heparin was the teratogenic agent. However, in this study, the heparin-plasma was successful at supporting embryonic development when in combination with FMP. This therefore suggests that the heparin alone is not harmful, but that there is a toxic effect when DMEM is combined with heparin-plasma. To determine whether it is the heparin or some other factor in the plasma which reacts with the DMEM, plasma would need to be prepared without using heparin. Proof that heparin was the aggrevating factor would be provided if embryos developed normally when cultured in this plasma in combination with DMEM. However, the major problem is that the only other anticoagulating agents are calcium chelating which cannot be used, as described previously.

As well as combining the plasma with FMP, the current study differed from Kesbys' study (1992) in a number of the steps taken to prepare the plasma. In both studies, the rats were anaethetised with diethyl ether for blood removal. Although 20 IU heparin per ml blood has been reported as the concentration required for the successful extraction of plasma from whole blood (Young & Bermes, 1986; Kesby, 1992), only 10 IU/ml was utilised in this study, resulting in only 5 IU/ml in the culture medium. In addition, the blood was added to a tube containing heparin-coated balls, rather than adding liquid heparin. Both the reduction in concentration, and the method of heparin application may have increased the suitability of heparin-plasma for embryo culture. The heparin-plasma was then extracted by centrifugation, although in Kesbys' study, the blood was only centrifuged for 5 minutes at 3500 rpm, in comparison to 2 centrifugations of 20 minutes and 10 minutes at 2400 rpm in this study to ensure that there was no accidental transfer of red blood cells. All samples were heated at 56°C for 30 or 40 minutes. However, Kesby does not appear to have gassed the plasma preparations, whereas in this study a gas mixture of 5% O_2, 5% CO_2, and 90% N_2 was introduced into the plasma. This was performed to displace the ether, which can be harmful to the embryos. Finally, in the current study, the plasma preparations were centrifuged twice at 14,000 rpm, for 15 minutes, removing the platelets and perhaps other components with adverse affects on embryonic culture. For example, fat globules were observed to have been displaced from the plasma as a result of the centrifugation.

All of these differences may have had an effect on the suitability of plasma for *in vitro* post-implantation embryo culture, but the major difference may be the reduction in the level of heparin used. Nevertheless, the data presented in this thesis suggests that plasma can be used to culture post-implantation embryos, but only in combination with FMP.

It was also noted in this study, that FMP, either in combination with rat serum or dCP, was more efficient than DMEM for culturing embryos *in vitro,* especially 8.5 dpc embryos.

4.3 Analysis of TGF-β1 Knockout Embryos.

TGF-β has been the focus of many studies over the years since its discovery as a factor capable of transforming normal rat kidney fibroblasts in culture (Delarco & Todaro, 1978). Its genetic, molecular, and biochemical properties have been analysed, and compared in different species, different tissues, and different cell types. Potential actions were also identified based on expression patterns and *in vitro* assays. However, any definitive roles and actions could not be known until the protein was analysed *in vivo*. This was recently performed by selectively disrupting the TGF-β1 gene by homologous recombination, thereby preventing translation of the wild-type, functional protein (Kulkarni *et al*, 1993). Analysis of the offspring identified that the major role of TGF-β1 in mice is in the control of the immune system. However, another critical role for TGF-β1 was identified in the embryos that could not survive gestation.

4.3.1 8.5 and 9.5 dpc TGF-β1 Knockout Embryos.

Preliminary studies on the identification of yolk sac abnormalities in the TGF-β1 knockout conceptuses were made by Dr.Marion Dickson (Dickson *et al*, 1995). These studies were extended by Dr.Dickson and myself, both working independently, but in a complementary fashion.

In the current study, embryos from TGF-$\beta1^{+/-}$ x TGF-$\beta1^{+/-}$ intercrosses were initially examined at 8.5 dpc. There was no observable phenotypic difference between TGF-$\beta1^{+/+}$, TGF-$\beta1^{+/-}$, or TGF-$\beta1^{-/-}$ embryos at this stage (Dickson *et al*, 1995). TGF-β1 gene expression is first detectable by RT-PCR at the pre-implantation stage (Rappolee *et al*, 1988), but all three TGF-β1 genotypes were present at 8.5 dpc, and in the expected Mendelian ratio of 1:2:1. This suggested that either TGF-β1 is not a prerequisite for development before or around the time of implantation, or that the pre-implantation embryo has a lower threshold requirement for TGF-β1, which is met by the oviduct and uterine levels of the maternal protein.

By *in situ* hybridisation, TGF-β1 is first detected at high levels at 7.5 dpc, within the blood islands of the yolk sac, the mesoderm of the allantois, and the cardiogenic mesoderm of the embryo, specifically the pro-angioblast progeniters (Akhurst *et al*, 1990). The exact function of TGF-β1 within these cells is not known, but it appears from the normal appearance of the TGF-$\beta1^{+/-}$ and TGF-$\beta1^{-/-}$ yolk sacs at 8.5 dpc that the protein is not critically involved in the progression of the blood islands to the corona. Neither the differentiation of the angioblasts in the cardiogenic mesoderm, or

the joining of the allantois to the ectoplacental cone was analysed in this study, although, by visual observation alone, there did not appear to be any defects in the formation of the chorio-allantoic connection.

At 7.5 dpc, the blood islands of the yolk sac contain haemangioblasts, which give rise to endothelial and haematopoietic cell lineages (Sabin, 1920; Wilt, 1965; Miura & Wilt, 1969). Haemangioblasts and both descendant lineages express TGF-β1 RNA from 7.5 dpc until much later in development (Akhurst *et al* , 1990). The TGF-β1 expressed in the blood islands may have a role in the formation of these cell types, and therefore have a role in haematopoiesis and vasculogenesis. This idea is also supported by the observation of expression of TGF-β1 in the blood cells in the foetal liver, and early embryonic endothelial cells (Heine *et al*, 1987; Lehnert & Akhurst, 1988; Gatherer *et al*, 1990; Akhurst *et al*, 1990).

Embryos from TGF-$\beta1^{+/-}$ x TGF-$\beta1^{+/-}$ matings were examined at 9.5 dpc, either direct from the uterus, or after 24 hours in culture. At this stage, some of the TGF-$\beta1^{+/-}$ and TGF-$\beta1^{-/-}$ embryos were distinguishable from their TGF-$\beta1^{+/+}$ littermates. The observable phenotypic differences in both cultured and non-cultured embryos were within the yolk sac, affecting either the vasculature or the red blood cells. Some of the yolk sacs contained only delicate and disorganised vessels, others had a reduced number of vessels, while others lacked vessels altogether. Varying degrees of anaemia were also observed in a number of yolk sacs. These defects were consistent with the expression pattern data for TGF-β1 activity in haematopoiesis and vasculogenesis, especially within the yolk sac (Akhurst *et al*, 1990), and would suggest a very localised (autocrine) mode of action for TGF-β1.

4.3.1.1 Formation of the Yolk Sac.

In the mouse, the yolk sac protects, oxygenates, and feeds the developing embryo. It starts to develop during the blastocyst stage when the embryo consists of the trophoblast, the blastocyst cavity, and the inner cell mass (ICM). The ICM differentiates into a bilaminar layer made up of primitive ectoderm and primitive endoderm. Cells from the endoderm form the parietal endoderm by migrating along the entire inner surface of the trophectoderm, and secreting Reichert's membrane (Gardner, 1982). The remainder of the primitive endoderm forms a layer of visceral endoderm around the egg cylinder. The yolk sac forms in the region where mesoderm, formed during gastrulation, is found apposed to the visceral endoderm. During development, this enlarges considerably, eventually residing against the parietal endoderm (Slack, 1983). At approximately 7.5 - 8 dpc, distinct regions of the

mesothelium are induced to proliferate by the underlying endoderm, and become the haemangioblasts of the blood islands (Miura & Wilt, 1969). The outer haemangioblasts differentiate into endothelial cells which anastamose to form blood vessels in a process called vasculogenesis. Angiogenesis occurs at a later stage by the sprouting of existing vessels. Primitive haematopoiesis also occurs in the yolk sac by the formation of blood cells from the inner haemangioblasts. There is evidence for and against the involvement of these primitive blood cells in definitive haematopoiesis which occurs in the foetal liver at a later stage (Moore & Metcalf, 1970; Medvinsky *et al*, 1993; Godin *et al*, 1993).

Molecular analysis of vasculogenesis has only recently been performed, and has grown rapidly with the advances in knockout methodology.

4.3.1.2 Molecular Aspects of Yolk Sac Vasculogenesis.

Vascularisation of the yolk sac is controlled by a variety of molecules. The extracellular matrix is important in the formation of vessels, enabling the endothelial cells to migrate, and form cohesive tubes. A transgenic knockout line was recently generated for the ECM protein fibronectin (FN), and was found to be embryonic lethal (George *et al*, 1993). On analysis of the embryos, it was discovered that development of the yolk sac vasculature stopped at the blood island stage and that the endodermal and mesothelial cell layers had separated. FN is, therefore, a prerequisite for the development of a normal vasculature. Certain cell surface receptors which interact with the ECM are also crucial. Removal of the $\alpha_V\beta_3$ integrin results in a phenotype similar to the FN knockouts, but disrupts angiogenesis, not vasculogenesis, and so the embryos survive to a slightly later stage (Brooks *et al*, 1994). The α5-integrin knockouts also develop blood vessels, but these vessels are weak, and the blood leaks into the yolk sac cavity. This "leakage" phenotype was also observed in the FN knockouts, and occasionally in the TGF-β1 knockouts (Yang *et al*, 1993; George *et al*, 1993; Dickson *et al*, 1995). The α5 mutants also fail to develop a functioning chorio-allantoic connection, with the result that intra-embryonic circulation is impaired.

Five receptor tyrosine kinases (RTK) which are almost solely expressed in the endothelial cell lineage have also been identified. The five RTKs can be divided into two groups, based on structural similarities (Hunter & Lindberg, 1994). The first group contains *Flt-4*, and the receptors for vascular endothelial cell growth factor (VEGF) *Flk-1* and *Flt-1* (Ferrara & Henzel, 1989; De Vries *et al*, 1992; Pajusola *et al*, 1992; Millauer *et al*, 1993), while the second group contains *Tie* and *Tek (=Tie-2)*

(Dumont *et al*, 1992, 1994; Sato *et al*, 1993). Expression analysis and knockout experiments suggest that each of these RTKs exerts a different effect on endothelial cells. Knockout experiments revealed that *Flt-1* is required for normal vessel assembly, but not for endothelial cell formation, whereas *Flk-1* seems to affect the haemangioblasts of the yolk sac, and is therefore required for blood island formation and vasculogenesis (Fong *et al*, 1995; Shalaby *et al*, 1995). *Tie* and *Tek*, although structurally similar, were also revealed to have different roles by knockout analyses (Sato *et al*, 1995). *Tie* is needed for the structural integrity of the endothelial cells, whereas *Tek* is required for angiogenesis.

As the expression data, and the phenotypes of the TGF-β1 knockout embryos examined in this study revealed, TGF-β1 appears to be another molecular control of yolk sac vasculogenesis. The appearance of the affected yolk sacs was surprisingly similar in the fibronectin (FN) (George *et al*, 1993) and some of the TGF-β1 knockouts. The development of the vascular system was arrested at the blood island/corona stage, and the endodermal and mesothelial cell layers were found to separate. This evidence suggests that TGF-β1 affects the ECM molecules in the yolk sac, which maintain the integrity of the yolk sac layers, and help to support the developing tubes. TGF-β is known to directly affect ECM, by controlling the production of certain ECM proteins, the degradation of the matrix, and the expression of integrins, but as yet, the composition of the ECM in the TGF-β1 knockouts has not been examined (Seyedin *et al*, 1985; Laiho *et al*, 1986; Varga *et al*, 1987; Ignotz & Massague, 1987; Pearson *et al*, 1988; Overall *et al*, 1989). FN production is directly affected by TGF-β1 *in vitro* (Ignotz & Massague, 1986; C.J. Roberts *et al*, 1988), and examination of the expression of this ECM molecule in the TGF-$\beta1^{-/-}$ yolk sacs in comparison to the TGF-$\beta1^{+/+}$ yolk sacs would reveal whether FN is affected in the TGF-β1 knockouts. It would also be interesting to analyse the converse, that is, whether the expression of TGF-β1 is affected by the depletion of FN in the FN knockouts. This would reveal whether the expression of TGF-β1 is indirectly affected by the composition of the ECM.

However, TGF-β1 is also known to act directly on endothelial cells in culture. Therefore, it is possible that the defective vessels result from the lack of the direct effect of TGF-β1 on endothelial cells. Previous studies have shown that in 2-dimensional culture, TGF-β1 inhibited the proliferation of endothelial cells (Baird & Durkin, 1986; Frater-Schroder *et al*, 1986). Conversely, TGF-β stimulated endothelial cells to differentiate and form tubes in a 3-dimensional collagen gel, without affecting their proliferation (Madri *et al*, 1988). These cells were also found to have increased

expression of FN, PECAM, and integrins, and they formed new tight cell junctions (Merwin *et al*, 1990; Basson *et al*, 1992; Madri *et al*, 1992).

To examine whether TGF-β1 affects the proliferation of endothelial cells *in vivo*, the current study utilised BrDU incorporation to analyse the proliferation rates of these cells in TGF-β1 knockout conceptuses. There was no significant difference in the proliferation rates of the endothelial cells in either the yolk sac or the embryo proper between TGF-$\beta1^{+/+}$ and TGF-$\beta1^{-/-}$ conceptuses. This suggested that endothelial cells are not growth inhibited by TGF-β1. Like the endothelial cells in the collagen gel, endothelial cells *in vivo* are supported on all sides. Therefore it is possible that TGF-β1, *in vivo*, does not affect endothelial cell proliferation, but rather differentiation (Madri *et al*, 1988). This could be examined in the TGF-$\beta1^{+/+}$ and TGF-$\beta1^{-/-}$ yolk sacs by analysing the expression of endothelial cell differentiation markers.

Any of the endothelial cell specific tyrosine kinase receptors can be used to determine the differentiative state of endothelial cells. *Flk-1*, one of the vascular endothelial cell growth factor receptors, is expressed at 7.0 dpc in the extraembryonic and embryonic mesoderm (Millauer *et al*, 1993; Dumont *et al*, 1995). When *Flk-1* was disrupted by homologous recombination, normal blood island formation did not occur in the yolk sacs of homozygote embryos, with the result that neither vessels or blood were able to form (Shalaby *et al*, 1995). The early expression of *Flk-1*, and the absence of blood islands or vessels in the knockout implicate a role for this TK receptor in the differentiation of the endothelial cells. The differentiative stage that the cells had reached was examined by analysis of the other endothelial cell receptor tyrosine kinases. The markers for endothelial precursors, *Flt-1*, *Flt-4*, and *Tie* were detectable in *Flk-1* homozygous embryos (Dumont *et al*, 1992; 1995; Kaipainen *et al*, 1995). However, *Tek*, a TK receptor involved in angiogenesis, and therefore, after terminal differentiation of the endothelial cells, was not detectable (Korhonen *et al*, 1994; Dumont *et al*, 1995; Sato *et al*, 1995). This suggested that there were endothelial precursor cells present in the *Flk-1* knockouts, but they were prevented from differentiating any further. An analysis of these TK receptors in the TGF-β1 knockout yolk sacs would indicate if the endothelial cell lineage had separated from the haemangioblasts at all, and if so, what stage of differentiation the cells had attained.

The phenotype of the *Flk-1* homozygote yolk sac was also similar to the TGF-β1 knockouts, with both haematopoiesis and vasculogenesis affected. However, it appears as if *Flk-1* has effect before TGF-β1. *Flk-1* expression is detected 0.5 dpc before TGF-β1, and the defective phenotype is observed approximately 1 day earlier, when the *Flk-1*$^{-/-}$ yolk sacs fail to develop blood islands (Millauer *et al*, 1993;

Dumont *et al*, 1995; Shalaby *et al*, 1995). However, preliminary expression studies performed by Dr.M.Dickson suggested that *Flk-1* expression is reduced in the TGF-$\beta 1^{-/-}$ yolk sacs at 8.5 dpc in comparison to TGF-$\beta 1^{+/+}$ yolk sacs (Dickson *et al*, 1995). As this did not reflect a reduction in endothelial cells as shown by BrDU analysis, it suggested that there was defective endothelial cell differentiation. *Flk-1* is expressed before TGF-β1, therefore the reduction in the number of *Flk-1* expressing cells suggests that TGF-β1 may be required to maintain the differentiative state of the cells. Although thorough quantititative studies are still to be performed, analysis of *Flk-1* enabled defective TGF-β1 knockout embryos to be detected before any morphological phenotype.

4.3.1.3 Molecular Aspects of Haematopoiesis

The development of the blood was also affected in a percentage of the TGF-$\beta 1^{+/-}$ and TGF-$\beta 1^{-/-}$ yolk sacs. As for vasculogenesis, the molecular aspects of haematopoiesis have only recently been investigated.

Primitive haematopoiesis occurs in the yolk sac at approximately 8-9 dpc, whereas definitive haematopoiesis occurs in the liver at approximately 14 dpc. There is controversy regarding the involvement of primitive blood cells in definitive haematopoiesis. Initial evidence suggested that the blood cells from the yolk sac migrated and formed a colony within the liver (Moore & Metcalf, 1970). However, more recently, the idea is that definitive haematopoiesis occurs independently from the yolk sac (Medvinsky *et al*, 1993; Godin *et al*,1993). This latter hypothesis is supported by the fact that each type is regulated by a separate group of molecules. Using knockout methodologies, GATA-1, GATA-2, and tal-1/SCL were all found to be involved in primitive haematopoiesis (Pevny *et al*, 1991; Weiss *et al*, 1994; Tsai *et al*, 1994; Shivdasani *et al*, 1995), whereas *dominant white spotting*, *steel*, *c-myb*, *retinoblastoma*, *c-kit*, erythropoietin, and the erythropoietin receptor gene products all regulate definitive haematopoiesis (Russell *et al*, 1968; Chui *et al*, 1974; Mucenski *et al*, 1991; Lee *et al*, 1992; Ogawa *et al*, 1993; Wu *et al*, 1995). There does not appear to be any cross-regulation, but all of these proteins appear to be crucial for haematopoiesis. Embryos that lack any of the above proteins do not complete gestation due to severe anaemia of either immature, nucleated yolk sac blood cells, or the mature liver blood cells.

As mentioned previously, a role for TGF-β1 in haematopoiesis was postulated from the expression of the RNA and protein in the haematopoietic lineages that descended from the haemangioblasts in the yolk sac (Akhurst *et al*, 1990). The TGF-β1

knockout embryos with defective yolk sacs were found to die at approximately 10.5 dpc, and both Dr.M.Dickson and I found that some of these yolk sacs were anaemic (Dickson *et al*, 1995). Therefore, TGF-β1 also appears to be involved in the regulation of primitive haematopoiesis. However, it is possible that the anaemia in the TGF-$\beta1^{+/-}$ or TGF-$\beta1^{-/-}$ yolk sacs is a secondary effect. Both foetal and adult haematopoiesis depend on a normal ECM, and stromal-cell interactions, and therefore the defective vasculature in some of the TGF-β1 knockouts may have affected haematopoiesis (Patel & Lodish, 1994; Rafii *et al*, 1994; Buzby *et al*, 1994). However, the appearance of the haematopoietic and endothelial defects in the TGF-β1 knockout yolk sac did not always coincide. Defective vasculature was observed in yolk sacs with an abundance of blood, and normal vasculature was present in yolk sacs which were anaemic. The presence of blood in the FN, and α5-integrin knockouts also supports the development of blood cells in an abnormal environment (George *et al*, 1993; Yang *et al*, 1993), even though previous studies had suggested that FN was a prerequisite for erythroid development (Patel & Lodish, 1987).

In the current study, although the anaemia was obviously due to a reduction in haemoglobinised cells, there were significant numbers of circulating cells in these yolk sacs. TGF-β1 was therefore affecting either the proliferation of the haemoglobinised cells, or the haemoglobinisation process itself. To investigate whether proliferation or differentiation was affected, Dr.M.Dickson analysed the expression of ζ-globin in 8.0 and 9.5 dpc conceptuses from TGF-β1 intercrosses (Wilkinson *et al*, 1987; Dickson *et al*, 1995). ζ-globin is a marker for the haemoglobinisation of the red blood cells, thereby indicating their differentiative state. No difference could be detected between the genotypes in embryos and yolk sacs of 8.0 dpc. At 9.5 dpc, it was found that the percentage of blood cells staining with the probe was identical in TGF-$\beta1^{-/-}$ and TGF-$\beta1^{+/+}$ yolk sacs. However, the total number of red blood cells hybridising with the probe in the TGF-$\beta1^{-/-}$ yolk sacs was up to 90% less than in the TGF-$\beta1^{+/+}$ yolk sacs. This suggested that the anaemic phenotype observed in the TGF-$\beta1^{-/-}$ yolk sacs resulted from a reduction in the total number of blood cells differentiating from the haemangioblasts.

One other interesting observation obtained from analysing the TGF-β1 embryos with anaemic yolk sacs was the presence of blood within the dorsal aorta at approximately 9.5 dpc. This was observed in Dr.M.Dicksons' studies, and both the *in vivo* and *in vitro* analyses in my own project. 9.5 dpc is prior to the formation of a functional foetal liver, and therefore, definitive haematopoiesis. It is possible that this blood represents the multipotential haematopoietic cells that arise in the paraaortic splanchnopleura (P-Sp) from 8.5-9.5 dpc (Godin *et al*, 1995). This group suggested

that there are two sites of haematopoiesis at 8.5 dpc, the yolk sac and the P-Sp. If this is true, then the results from the TGF-β1 knockouts suggest that TGF-β1 is only involved in yolk sac haematopoiesis. Molecular analysis of other controlling factors, such as GATA-1 and GATA-2, in the P-Sp blood would determine whether it was distinct from both yolk sac, and liver haematopoietic events.

4.3.1.4 The Role of TGF-β1 in Vasculogenesis and Haematopoiesis.

Although the yolk sac as the initial site of the effect of TGF-β1 deficiency is in agreement with previous expression studies (Akhurst *et al*, 1990), the actual effect itself is inconsistent with the postulated roles for the protein. As TGF-β1 was suggested to be a potent inhibitor of endothelial and haematopoietic cells, it was expected that there would be over-production of red blood cells and hyperplasia of the endothelial cells (Heimark *et al*, 1986; Muller *et al*, 1987; Ohta *et al*, 1987; Ottman & Pelus, 1988). However, as discussed, there was, in fact, a decrease in the number of red blood cells, and no effect on the proliferation of the endothelial cells. This data suggests that the effect of TGF-β1 *in vivo* is in the control of haematopoietic and endothelial cell differentiation. It may also be that the phenotypes of the TGF-β1 knockout yolk sacs were actually an indirect effect of lack of TGF-β1, such as modulation of the ECM, or of the expression of other growth factors.

4.3.1.5 Phenotype of the TGF-β1 Knockout Embryos.

In this study, and the analyses performed by Dr.M.Dickson, the morphological appearance of the embryo *per se* at 9.5 dpc was surprisingly normal in all genotypes, whether the yolk sac was normal or abnormal. There was a slight delay in development, approximately 0.5 dpc, and a little necrosis in the embryos with defective yolk sacs, but this was likely to be an effect of yolk sac insufficiency. On analysis of the limited embryonic vasculature which had developed by this stage, the structure of the vessels appeared normal. This was a surprising result as high levels of TGF-β1 have been detected in these endothelial cells (Akhurst *et al*, 1990). It is possible that the endothelial cells within the embryo are more sensitive to TGF-β1, and therefore lower levels are required to elicit a response. The maternal TGF-β1 (TGF-$\beta1^{+/-}$) crossing the placenta may be sufficient for the embryonic endothelial cells, but insufficient for the yolk sac cells. Indeed, one group observed that when the embryos were not exposed to maternal TGF-β1 by gestation in a TGF-$\beta1^{-/-}$ female, the TGF-$\beta1^{-/-}$ offspring had severe cardiac abnormalities (Letterio *et al*, 1994). When Dr.M.Dickson examined 3 12.5 dpc TGF-$\beta1^{-/-}$ embryos from a TGF-$\beta1^{-/-}$ mother, abnormal heart development was also observed (Dickson *et al*, 1995). However, each

of these embryos also exhibited a defective yolk sac, and therefore, the heart defects may have been a secondary defect. Ventricular hyperplasia and hypertrophy can result from defective pressure in the cardiovascular system (Heine *et al*, 1985).

It is also possible that TGF-β1 only exerts an effect on the endothelial cells of the yolk sac, and that the expression in the embryonic endothelial cells is non-functional. The Zebrafish mutation, *cloche*, influences both endothelial and haematopoietic cell lineages, but only the endothelial lining of the heart, the endocardium, is affected (Stainier *et al*, 1995). The identity of the mutated protein which results in this phenotype is not yet known.

Due to embryonic death occuring at approximately 9.5-11.5 dpc, it was impossible to identify any other defects which may have occured at a later stage of development as a result of the lack of TGF-β1. This included definitive haematopoiesis, although at 9.5-10.5 dpc Dr.M.Dickson noted that the liver appeared to be developing normally (Dickson *et al*, 1995).

4.3.2 Autocrine or Paracrine Mode of Action.

Analysis of mRNA and protein expression patterns in the embryo suggest that the TGF-βs may have both paracrine, and autocrine modes of action (Millan *et al*, 1991; Pelton *et al*, 1991). For example, in many sites of epithelial-mesenchymal interactions, such as in the developing salivary glands, tooth, and heart, there is mirror-image expression of the mRNA in the epithelium, and the protein in the mesenchyme, suggesting that there is paracrine activity (Akhurst, 1994). However, there are also sites where the mRNA and protein are detected in the same cells, for example, in cardiomyocytes (Dickson *et al*, 1993).

In the TGF-β1 yolk sac, if the defective vasculature was as a result of disrupted ECM, this would strongly support a paracrine activity for the TGF-β produced in the yolk sac endothelial cells. However, as TGF-β1 RNA is expressed in the haemangioblasts, the endothelial cells, and the haematopoietic cells (Akhurst *et al*, 1990), and these are the cells affected in the abnormal yolk sacs, this suggests that the mode of TGF-β1 action is autocrine. Determination of the site of TGF-β1, and TGF-β receptor proteins in the yolk sac would help to elucidate whether the protein acts through a paracrine, or an autocrine activity. If they were present in the ECM, then it would be a paracrine activity, whereas if they were present in the endothelial and haematopoietic cells, this would indicate an autocrine activity.

As mentioned above, within the embryo proper the endothelial cells did not appear to be affected by the lack of TGF-β1 in the knockouts. Previously, expression studies had found that TGF-β1 mRNA was expressed in the endothelial cells (Akhurst *et al*, 1990), but the protein was not (Pelton *et al*, 1991). This would suggest a paracrine mode of action for the TGF-β1 produced by these cells, which would explain the lack of abnormal phenotype in the vasculature of the embryos in the TGF-β1 knockouts. The target for the protein produced by these cells is unknown.However, it is possible that the effect of removing this TGF-β1 would have been identified at a later stage, if the embryos had survived. But it is also possible that the protein is non-functional. Nevertheless, these endothelial cells did not appear to be affected by the lack of TGF-β1 at 9.5 dpc. Again, analyses of the receptor expression patterns would help to elucidate whether the TGF-β1 produced by the embryonic endothelial cells has an autocrine, or paracrine activity, if any activity at all.

4.3.3 The Rescue and Range of Phenotypic Embryos.

As discussed, a wide range of phenotypes were observed in the TGF-β1$^{-/-}$ embryos. Either they were defective, with abnormal yolk sac vasculature, or anaemia, or both, or they were perfectly normal and the mice survived to approximately 3 weeks post-partum. The percentage of TGF-β1$^{-/-}$ embryos that survived gestation may have been able to do so as a result of maternal transfer of TGF-β1, as shown by ^{125}I-TGF-β transmittance from mother to pup (Letterio *et al*, 1994). This hypothesis was also supported by the observation that TGF-β1$^{+/-}$ embryos from TGF-β1$^{+/-}$ intercrosses were rescued after being transferred into a TGF-β1$^{+/+}$ mother (Dickson *et al*, 1995).

In this project the role of maternal TGF-β1 was investigated by the removal and culture of 8.5 dpc embryos from TGF-β1 intercrosses in a medium lacking TGF-β. If maternal TGF-β1 was responsible for rescuing the embryos, it was expected that there would be an increase in the number of defective embryos in the TGF-β1-depleted environment. There was no increase in abnormalities. However, this does not conclusively indicate that maternal TGF-β1 is not rescuing some of the knockout embryos. Although the embryos were removed before the appearance of any phenotypic differences, maternal TGF-β1 may have already exerted its affect by this stage. As described previously, there is an observable difference in *Flk-1* expression in the abnormal TGF-β1$^{-/-}$ yolk sacs before the appearance of any defects. Maternal TGF-β1 may also have been bound to the ECM in the embryos, and thereby transferred over into the culture system.

As well as maternal TGF-β1, the other TGF-β isoforms may also be involved in the rescue of the TGF-β1$^{-/-}$ embryos. Examination of endogenous TGF-β2 and TGF-β3 in the embryo and the yolk sac showed that they were not up-regulated. However, it is possible that the maternal isoforms are involved. TGF-β2 is unlikely to be responsible since neither endothelial or haematopoietic cells respond to this isoform (Ohta *et al*, 1987; Ottman & Pelus, 1988; Merwin *et al*, 1991; Qian *et al*, 1992). However, maternal TGF-β3 may have a role (Greycar *et al*, 1989; Cheifetz *et al*, 1990). Analysis of TGF-β3 levels, especially in TGF-β1$^{-/-}$ mothers which give birth to TGF-β1$^{-/-}$ pups, would help to determine whether this factor is involved in the rescue of the abnormal phenotypes.

None of the above hypotheses however explain why only a percentage of TGF-β1$^{-/-}$ embryos are rescued. It appears that even though individual embryos may have been of the same genotype, there were other genetic variabilities which affected their phenotype. Other transgenic knockout mice, such as the fibronectin mice, exhibited a variable phenotype, which has been ascribed to genetic polymorphisms as a result of the use of a mixed genetic background (George *et al*, 1993). The TGF-β1 knockouts analysed in the current study were also on a mixed genetic background (50% NIH, 37.5% C57/Bl6, 12.5% 129sv). Therefore, polymorphisms in modifier genes of TGF-β1 may be responsible for both the percentage of embryonic rescue, and the precise defects observed in the individual conceptuses. The gene(s) affected may include those involved in the control of circulating maternal TGF-β1, or genes that would affect the ability of the embryos to respond to or take-up maternal TGF-β1. Preliminary investigations by others in the laboratory have indeed provided compelling evidence for genetic factors influencing the outcome of the TGF-β1 phenotype. When analysed on a 93% C57Bl6 genetic background there is complete (100%) prenatal lethality of TGF-β1$^{-/-}$ animals, whereas on a 93% NIH genetic background there is only <15% prenatal lethality. Genetic breeding experiments have already shown a genetic linkage between prenatal lethality at one major locus (Bonyadi & Akhurst, pers. comm.).

One example of a defect resulting from a mutation in a gene involved in the response to TGF-β is the hereditary disorder haemorrhagic telangiectasia type 1, the effects of which include vascular dysplasia, and recurrent haemorrhage. Endoglin, a TGF-β type III receptor, is thought to be a likely candidate gene for the disease (McAllister *et al*, 1994). The type III receptors do not appear to be necessary for TGF-β signalling (Cheifetz *et al*, 1990) but disruption of endoglin would probably affect TGF-β binding to the signalling receptors. Although this is indirect evidence, it also supports

the role for TGF-β in vascularisation, a role that was strongly implicated in the current study.

4.3.4 Conclusions.

The major outcome of this study was that the abnormal phenotype resulting from removal of functional TGF-β1 was in full accordance with its initial site of high expression in the yolk sac, and its postulated role in haematopoiesis and vasculogenesis. The data also suggested that the role of TGF-β1 *in vivo* is not as a endothelial and haematopoietic growth inhibitor, but rather as a regulator of endothelial differentiation and haematopoiesis. Whether TGF-β1 acts directly on these cells, or indirectly through the modulation of the expression of other growth/differentiation factors, or the ECM is not yet known. Analysis of the expression of ECM proteins, and differentiation markers will help to elucidate the defective phenotypes observed in the TGF-β1 knockout conceptuses, and also reveal the mode of action of TGF-β1 on endothelial and haematopoietic cells of the yolk sac.

Further investigations are also required to determine the reason for the dichotomy in embryonic survival. As mentioned in 4.3.3, genetic polymorphisms may be responsible, possibly affecting maternal TGF-β1 circulatory levels, or the ability of the embryos to take-up or respond to maternal TGF-β1. Maternal levels could be examined by performing CCL64 assays on serum samples from females of different genotypes, while the ability of the embryo to respond could be analysed by examining the expression of the TGF-β receptors, and the intracellular signalling systems.

APPENDIX A

STATISTICAL DATA.

Amendment to Statistical Data.

It should be noted that due to the large sample sizes, and therefore the large number of students t-tests performed using individual values, only the P-values in close proximity of 0.0001 should be considered as significant. Further statistical tests utilising the data presented here would determine those P-values that indicate true statistical significance.

	FMP : 50% rat serum	FMP : 10% rat serum	FMP : 50% dCP	FMP : 10% dCP	DMEM : 50% rat serum	DMEM : 10% rat serum	DMEM : 50% dCP
FMP : 50% rat serum							
FMP : 10% rat serum	0.45						
FMP : 50% dCP	0.032	0.038					
FMP : 10% dCP	0.047	0.0004	0.0000				
DMEM : 50% rat serum	0.73	0.61	0.024	0.0047			
DMEM : 10% rat serum	0.80	0.058	0.0007	0.0083	0.36		
DMEM : 50% dCP	0.019	0.0000	0.0000	0.76	0.0006	0.0005	
DMEM : 10% dCP	0.054	0.002	0.0000	0.84	0.0041	0.0058	0.57

Appendix A1 - Embryonic Scoring Data for 7.5 dpc Embryo Cultures.

7.5 dpc embryos were cultured in a variety of media for 24 hours before morphological analysis. Their development was scored using the Brown-Fabro method (1981). To determine whether any of the media were more supportive of embryonic development, the scores of embryos cultured in the different media were compared by a 2-sample students t-test. The results are shown above. A statistically significant difference was indicated by a P-value $\leq$ 0.05.

As mentioned in the Results section, this data should be considered in conjunction with the number of embryonic abnormalities resulting from culture in each medium.

	FMP : 50% rat serum	FMP : 10% rat serum	FMP : 50% dCP	FMP : 10% dCP	DMEM : 50% rat serum	DMEM : 10% rat serum	DMEM : 50% dCP
FMP : 50% rat serum							
FMP : 10% rat serum	0.10						
FMP : 50% dCP	0.88	0.17					
FMP : 10% dCP	0.068	0.72	0.11				
DMEM : 50% rat serum	0.0076	0.045	0.010	0.0069			
DMEM : 10% rat serum	0.025	0.32	0.042	0.54	0.15		
DMEM : 50% dCP	0.0001	0.0009	0.0001	0.0020	0.47	0.0079	
DMEM : 10% dCP	0.30	0.56	0.41	0.38	0.026	0.16	0.0004

Appendix A2 - Embryonic Scoring Data for 8.5 dpc Embryo Cultures.

8.5 dpc embryos were cultured in a variety of media for 24 hours before morphological analysis. Their development was scored using the Brown-Fabro method (1981). To determine whether any of the media were more supportive of embryonic development, the scores of embryos cultured in the different media were compared by a 2-sample students t-test. The results are shown above. A statistically significant difference was indicated by a P-value ≤ 0.05.

As mentioned in the Results section, this data should be considered in conjunction with the number of embryonic abnormalities resulting from culture in each medium.

	FMP : 50% dCP (33.63)	FMP : 10% dCP (29.30)
FMP : 50% dCP (33.63)		
FMP : 10% dCP (29.30)	0.022	
DMEM : 50% dCP (19.93)	0.0000	0.0000

Appendix A3 - In-depth Analysis of 8.5 dpc Embryo Cultures.

A further twenty-six or twenty-seven 8.5 dpc embryos were cultured in each of the following media; FMP : 50% dCP, FMP : 10% dCP, and DMEM : 50% dCP. After culture, the embryos were scored by the Brown-Fabro method (1981). The mean score for each medium is shown in brackets. To determine whether there was a statistically significant difference between the scores of the embryos cultured in the different media they were compared by a 2-sample students t-test. The results are shown above. A statistically significant difference was indicated by a P-value ≤ 0.05.

As mentioned in the Results section, this data should be considered in conjunction with the number of embryonic abnormalities resulting from culture in each medium.

	FMP : 50% dCP (777/2000)	FMP : 10% dCP (790/2000)	DMEM : 50% dCP (429/2000)
FMP : 50% dCP (777/2000)			
FMP : 10% dCP (790/2000)	0.73		
DMEM : 50% dCP (429/2000)	0.0000	0.0000	
Control Embryos (638/2000)	0.0025	0.0003	0.0001

Appendix A4 - BrDU Incorporation in Cultured 8.5 dpc Embryos.

Embryos in culture, or control embryos were injected with 1µl 10mmol/l BrDU (See Materials & Methods). This was performed to assess the proliferation rates of the embryos, and thereby determine whether there was any variation in growth between the embryos cultured in different media. After immunohistochemistry, the number of cells stained for BrDU was visible. A total of 2000 ectodermal cells surrounding the embryos were counted, and the number of stained cells noted (figures in brackets). The data was statistically compared between the different media using a 2-sample students t-test, and the results are shown in the table above. A statistically significant difference is indicated by a P-value $\leq$ 0.05.

	FMP : 50% dCP (276)	FMP : 10% dCP (229.7)	DMEM : 50% dCP (170.7)
FMP : 50% dCP (276)			
FMP : 10% dCP (229.7)	0.17		
DMEM : 50% dCP (170.7)	0.0024	0.0079	
Control Embryos (387)	0.21	0.092	0.034

Appendix A5 - Protein Concentrations in Cultured Embryos.

As described in the Materials & Methods section, the protein was extracted from each cultured embryo using the Lowry method (1951). The concentration of protein reflects the developmental stage and condition of the embryos after culture. Therefore, to determine whether there was a statistically significant difference between the embryos cultured in each medium, the protein values for each culture group were statistically analysed by 2-sample students t-tests. A resulting P-value$\leq$ 0.05 indicates a statistically significant difference. The results are presented in the table above.

The numbers in brackets represent mean protein concentrations for the embryos cultured in that medium.

	Stained vs Total Number of Cells Counted in TGFβ1$^{+/+}$	Stained vs Total Number of Cells Counted in TGFβ1$^{-/-}$
All Yolk Sac Cells	1056 / 3041	1047 / 3036
P-Value	0.94	
Mesothelial + Endothelial Cells	935 / 2160	1028 / 2700
P Value	0.22	
Endodermal Cells	1418 / 2413	932 / 2800
P Value	0.29	
Endothelial Cells	586 / 1735	459 / 1541
P Value	0.18	

Appendix A6 - BrDU Incorporation in the Yolk Sacs of TGF-β1 Knockout Embryos.

To determine whether there was a difference in cell proliferation rate in the TGF-β1$^{+/+}$ and TGF-β1$^{-/-}$ embryos, 1μl 10mmol/l BrDU was injected into the yolk sac cavity before culture for 1 hour in FMP : 50% dCP (See Materials & Methods). After immunohistochemistry, the number of cells stained for BrDU was visible. A range of cell types were analysed in the yolk sac with a total of approximately 3000 cells counted per cell type, per genotype, as shown above. The data was statistically compared between the different genotypes using 2-sample students t-tests. A P-value ≤ 0.05 represents statistical significance.

	Stained vs Total Number of Cells Counted in TGFβ1$^{+/+}$ Embryos	Stained vs Total Number of Cells Counted in TGFβ1$^{-/-}$ Embryos
Surrounding Epithelial Cells	958 / 3000	922 / 3000
P Value	0.17	
Neural Tube Epithelial Cells	953 / 3000	907 / 3000
P Value	0.29	

Appendix A7 - BrDU Incorporation in TGF-β1 Knockout Embryos.

To determine whether there was a difference in cell proliferation rate in the TGF-β1$^{+/+}$ and TGF-β1$^{-/-}$ embryos, 1μl 10mmol/l BrDU was injected into the yolk sac cavity before culture for 1 hour in FMP : 50% dCP (See Materials & Methods). After immunohistochemistry, the number of cells stained for BrDU was visible. Two epithelial cell types were analysed in the embryo proper, with a total of 3000 cells counted per cell type, per genotype. The data was statistically compared between the different genotypes using 2-sample students t-tests, and the results are shown above. A P-value ≤ 0.05 represents statistical significance.

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