S100A8 in Development

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Abstract

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S100 proteins are a family of Ca^{2+} binding EF-hand proteins. S100A8 is a cytosolic protein expressed in myeloid cells and epithelia where it forms a stable heterodimer with another S100 protein family member, S100A9. The S100A9 null mouse is viable and has no gross defect whereas the S100A8 null mouse is embryonic lethal. It was originally proposed that the S100A8 null mouse is lethal at E 9.0 in development due to lack of expression at E 6.5 in ectoplacental cone cells. This thesis shows that the S100A8 null phenotype is more complex than originally thought. S100A8 has a role in preimplantation development, which is previously unstudied. A small number of S100A8 null embryos survive to blastocyst but none survive implantation showing fatal compromise of S100A8 null embryos early in development. This thesis presents evidence that this lethality presents between fertilisation and E 2.5 of development. S100A8 also has a role in the murine decidua after implantation possibly key to normal murine development. S100A8 mRNA is highly expressed in maternal decidua yet S100A8 protein is not highly expressed. Foetal yolk sac cells do not express S100A8 mRNA yet they do stain positively for S100A8 protein. This thesis proposes that S100A8 protein is generated in the murine decidua and exported to the foetus where haematopoietic cells present the protein. The S100A8 protein has been shown to be expressed and stable independently of its myeloid partner, S100A9. These observations explain the discrepancy between the two S100 null mouse phenotypes and add new insight to the S100A8 null phenotype.

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Abbreviations

AA	Arachidonic acid
Ab	Antibody
ABC	Avidin-biotin complex
AGM	Aorta-gonads-mesonephros
AJ	Adherens junction
Asp	Aspartic acid
AQP	Aquaporin
bFGF	Basic fibroblastic growth factor
BMP	Bone morphogenic protein
BSA	Bovine serum albumin
cDNA	Complementary DNA
DAB	3,3'-diaminobenzidine
d.p.c	Days post coitus
E	Embryonic stage
EBs	Embryoid bodies
EDTA	Ethylenediaminetetraacetic acid
EFS	Embryonic feed cells
EPI	Epiblast
EtOH	Ethanol
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate,
fMLP	N-Formylmethionyl-leucyl-phenylalanine
Glu	Glutamine
H	Helix
HMWK	High molecular weight kininogen
Hr	Hour
HRP	Horseradish peroxidase
HSC	Haematopoietic stem cell
Ig	Immunoglobulin
LIF	Leukaemia inhibitory factor
LPS	Lipopolysaccharide
mAb	Monoclonal antibody

MIF	Migration inhibitory factor
Mins	Minutes
MIP	Macrophage inflammatory protein
MMP	Matrix metalloproteinase
mRNA	Messenger RNA
MRP	MIF related protein
MTG	Monothioglycerol
Mw	Molecular weight
NADPH	Nicotinamide adenine dinucleotide phosphate
NBF	Neutral buffered formalin
NFκB	Nuclear factor kappa B
NIF	Neutrophil inhibitory factor
NP	Neuropilin
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PE	Primitive endoderm
PLCβ	Phospholipase C beta
PMA	Phorbol 1-12-myristate-13-acetate
pSP	Para splanchnopleuria
RNAi	RNA interference
SDS PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
TAE	Tris acetate
TIMPS	Tissue inhibitors of metalloproteinases
UTR	Untranslated region
V	Volts
VEGFR	Vascular endothelial growth factor receptor
ZP	Zona pellucida

CHAPTER 1

1 Introduction

1.1 S100 Proteins

The S100 proteins are a subfamily of the EF-hand Ca^{2+} binding proteins. They are named S100 due to their solubility in 100% ammonium sulphate at neutral pH. The first members of the family were discovered in the S100 fraction of bovine brain homogenate and were originally named S100A and S100B (Moore 1965). There are now approximately 20 S100 family members and they will be referred to throughout this thesis using their updated nomenclature (Marenholz, Lovering et al. 2006).

The S100 family are low weight (8-13kD), acidic proteins, which mostly form either homo or heterodimers (Marenholz, Heizmann et al. 2004) (Donato 2001). They contain two EF hand motifs, a classical 12-residue C-terminal EF hand and an atypical 14-residue N terminal EF hand, which is specific to the S100 protein family. The EF hands are flanked by hydrophobic termini and connected by a central hinge region as shown in fig 1.1.

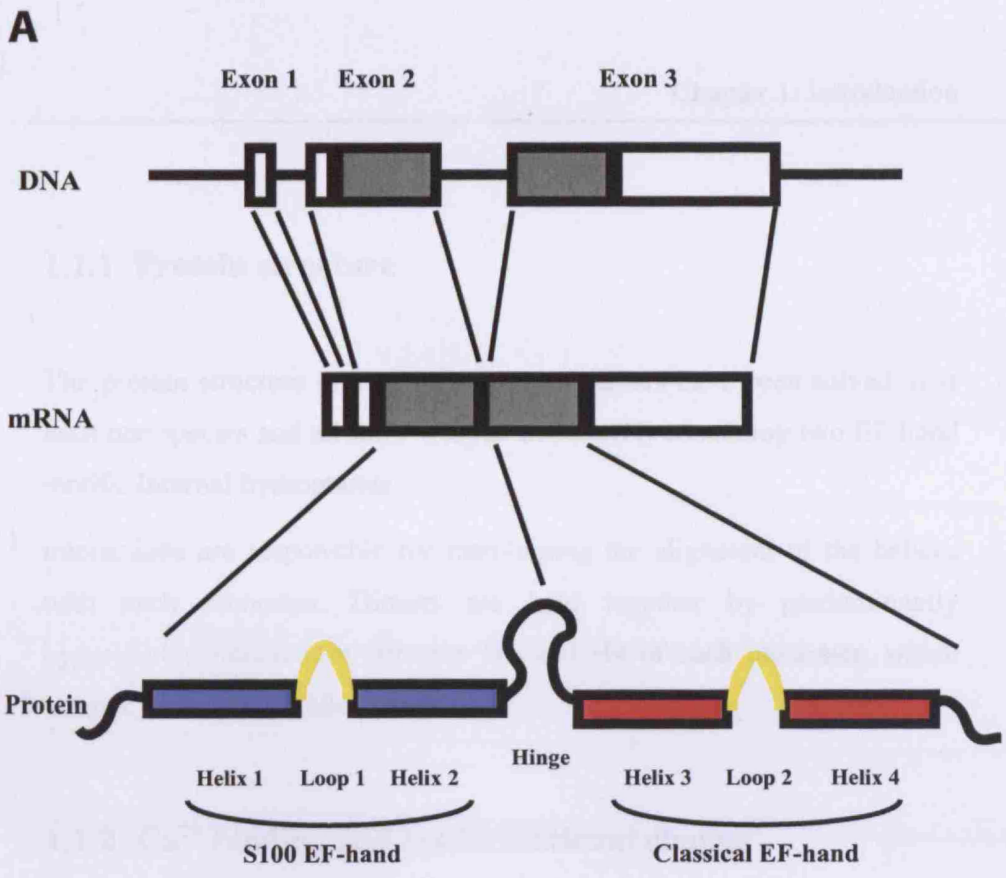


Figure 1.1 S100 gene, mRNA and protein structure with S100 gene insertion strategy. Diagram of typical S100 family gene structure and the mRNA produced from the gene (A) Sequences expressed in the protein are shaded grey while untranslated regions are white. 3) Typical protein structure of S100protein. EF hands are represented by coloured boxes (alpha helices) and calcium binding loops in yellow. Areas with no secondary structure are shown as black lines. Typical insertion strategy used to silence S100 genes in this thesis (B) Neo insert (Green) sits within the sequence of Exon 2 just after the start codon (Blue).

1.1.1 Protein structure

The protein structure of 13 S100 family members have been solved in at least one species and all show 4 alpha helices (H) containing two EF-hand motifs. Internal hydrophobic

interactions are responsible for maintaining the alignment of the helices with each monomer. Dimers are held together by predominantly hydrophobic interactions between H1 and H4 of each monomer, which arrange in an anti-parallel orientation.

1.1.2 Ca^{2+} binding and conformational change

All S100 proteins bind Ca^{2+} with the exception of S100A10. The structures of apo and Ca^{2+} bound S100 proteins have been solved for many S100 proteins and show conformational change on binding Ca^{2+} (Drohat, Amburgey et al. 1996; Drohat, Baldisseri et al. 1998; Otterbein, Kordowska et al. 2002). The two different EF hands within an S100 family protein often have different Ca^{2+} binding affinities with the C–terminal hand having the higher affinity (Gribenko and Makhatadze 1998). Ca^{2+} binding causes global shape changes and leads to the exposure of a hydrophobic binding patch. Typically helices 1,2 and 4 remain largely unchanged while helix 3 reorients from being parallel to helix 4 to almost perpendicular to it. The hinge region moves from the interior of the protein to the exterior. This reorientation exposes the hydrophobic patch formed largely from the hinge region, Helix 3 and 4 and the C-terminal region. This conformation is often called the open conformation. In an open S100 homodimer two patches are formed which are symmetric whereas with an S100 heterodimer the patches are asymmetric.

This Ca^{2+} induced conformational change is strong evidence of a Ca^{2+} sensor role in S100 proteins. Also because of their lack of identified functional domains, it is generally believed that S100 proteins act through protein-protein interactions mediated by the hydrophobic patches they display in open conformation (Heizmann and Cox 1998). The *in vitro* Ca^{2+} binding affinity of most S100 proteins (μM to mM) lies outside the physiological range of Ca^{2+} within the cell (100 nM to 1 μM) (Heizmann and Cox 1998) yet it is thought that *in vivo* S100 proteins can be influenced by other factors including binding partners, Zn^{2+} and highly localised Ca^{2+} concentrations. It should also be noted that some S100 proteins have lost the ability to bind Ca^{2+} either in one EF hand as is seen with S100A7 (Brodersen, Nyborg et al. 1999) and S100A11 (Rety, Osterloh et al. 2000), or both EF hands as with S100A10 (Rety, Sopkova et al. 1999). However this does not stop the adoption of the open, "Ca^{2+} bound" conformation. S100A7 can still switch to the open conformation and S100A10 is constitutively in the open, "Ca^{2+} bound" conformation despite not binding Ca^{2+}.

1.1.3 Target interaction

The S100 hydrophobic patch is shallow and flat and target binding is highly specific. The binding region is largely helical and formed of basic and hydrophobic residues. The target sequence is typically acidic and hydrophobic in nature. Crystals of several S100 proteins with peptides (Rety, Sopkova et al. 1999; Rety, Osterloh et al. 2000; Rustandi, Baldisseri et al. 2000) bound have shown that there is no single orientation of peptide binding within the hydrophobic patch. Peptides such as p53 and NDR, crystallised separately with S100B, bind almost perpendicular to each other within the same hydrophobic patch (Bhattacharya, Bunick et al.

2004). It is possible that with proteins a broad attachment is possible in the binding site. Close inspection shows that the binding sites of S100 proteins and known target peptides show complementary electrostatic residues bringing hydrophobic residues into close proximity (Bhattacharya, Bunick et al. 2004). The variation of S100 protein sequence within the hydrophobic patch should therefore explain the variety of target proteins capable of binding.

1.1.4 Zn^{2+} and Cu^{2+}

Some S100 proteins are also capable of binding Zn^{2+} and Cu^{2+} at a site distinct from the Ca^{2+} binding sites. Zn^{2+} has been shown to bind to S100A2, A3, A5, A6, A7, A9, A12 and B (Heizmann and Cox 1998) and binding can increase or decrease Ca^{2+} affinity. Several S100 proteins have Zn^{2+} binding motifs and tertiary structures give rise to clusters of Zn^{2+} binding motifs strongly suggesting a distinct binding site (Clohessy and Golden 1996). Zn^{2+} binding sites in S100 proteins are frequently found near the dimer interface and it is thought that Zn^{2+} binding may stabilise dimer formation. It must be remembered though that Zn^{2+} is present in the cell at low concentrations (<0.1 nM) so S100 protein interactions with Zn^{2+} may be dependent on localisation to high Zn^{2+} vesicles or the extracellular milieu. Cu^{2+} also binds to several S100 proteins and can be displaced by Zn^{2+} suggesting mutual binding sites (Nishikawa, Lee et al. 1997).

1.1.5 Gene evolution and structure

S100 proteins evolved comparatively recently and have so far been found only in vertebrates. No S100 like gene has been found in worms, flies or protozoa despite the fact that they do contain other EF hand genes like

calmodulin and troponin. Despite their recent emergence S100 proteins are the largest sub family of the EF hand group suggesting rapid expansion of S100 genes from a single ancestor (Zimmer, Chessher et al. 1996). There are currently 20 recognised human S100 proteins, which vary in homology between 22% and 57% at the gene level. Across species individual S100 genes show a high degree of homology with above 75% identity within mammalian genes and even Xenopus show higher than 50% homology. At the amino acid level there is also a high degree of homology for individual S100 genes across species. Homology at this level can be as high as 50% in mammals (Zimmer, Chessher et al. 1996).

There is a high conservation both of gene structure and chromosomal arrangement between human, rat and mouse. There are S100 gene clusters on human chromosome 1 (Schafer, Wicki et al. 1995), mouse chromosome 3 (Ridinger, Ilg et al. 1998) and rat chromosome 2 with both gene direction and position being highly conserved leading to the numerical nomenclature of most S100 proteins (Schafer, Wicki et al. 1995). There are also S100 genes not located to these main clusters such as S100B, S100G, S100P and S100Z in human and mouse. These genes are assigned a letter in the nomenclature to reflect that they would not fit into the numerical system from the main cluster. Within the clusters there are also discrepancies in arrangement. Human S100A1 and S100A13 are found together yet in mice they are separated. This is, however, consistent with known chromosomal rearrangements in this area in mouse (Ridinger, Ilg et al. 1998). In human there are 4 main subgroups by homology (Marenholz, Heizmann et al. 2004), which appear to be evolutionarily linked suggesting multiple gene duplication events. Of interest is the S100A8, S100A9 and S100A12 group, which are clustered within 30Kb although not all subgroups are clustered together.

The exon-intron structure of S100 genes is also highly conserved with a distinctive 3 exon 2 intron template as shown in fig 1.1. The first exon contains the 5' UTR region, the second exon contains the N-terminal EF hand and the third exon contains the C-terminal EF hand and the 3'UTR. The only exception to this is S100A5, which contains 4 exons and 3 introns. Exon 1 contains 5'UTR in S100A5 and is similar to a typical exon 1. Exon 2 is similar to exon 1 with 5' UTR dominating with a start site and 13 residues of particularly long N-terminal sequence contained at the 3' end. Exon 3 contains the majority of the protein and exon 4 contains the C-terminus and 3' UTR. The promoter, intron, 5' and 3' UTR regions show little homology between S100 genes although first intron length is highly conserved. The lack of homology in these regions indicates that co-regulation is unlikely and that the S100 genes are regulated differentially. This is consistent with their individual patterns of expression.

1.2 Expression

S100 proteins are characterised by their cell and tissue specific expression, which is summarised in table 1.1.1. Some S100 proteins are widely expressed such as S100A4 and S100A6, whereas others are highly restricted e.g. S100P. Some cells contain multiple S100 proteins, for example, murine glial cells contain S100B, S100A1, S100A6 and S100A4 (Nishiyama, Takemura et al. 2002). S100 expression can be inducible or can be regulated during processes such as cell differentiation and cell cycle progression (Kligman and Hilt 1988; Zimmer and Landar 1995). The varied expression pattern confirms the highly specialised nature of the S100 proteins and reinforces the idea that their functions are varied

1.2.1 General functions

There are many proposed functions for the S100 proteins and they are summarised in table 1.1.1 Some S100 proteins have yet to have a function proposed for them indicating that work on the family is far from even or comprehensive. Common themes to emerge from the family include Ca^{2+} modulation, chemo-attraction, anti-microbial activity, cytoskeletal interaction and cell differentiation. Some of the functions described are intracellular and some are extracellular. S100 proteins lack export signals but it is thought that they may be secreted via a novel pathway (Rammes, Roth et al. 1997). It should be noted that most of the evidence for S100 functions come from *in vitro* studies and the exact physiological role of most S100 proteins is unclear.

1.2.2 S100 proteins in disease

S100 proteins have been associated with a number of disease states such as cancer, heart disease, inflammatory disorders and neurodegeneration. In many of these disease states it is still unknown whether unusual S100 expression is a cause or effect of the underlying condition.

1.2.3 S100 proteins in cancer

In cancer S100 proteins like S100B, S100A2, S100A4, S100A7 and S100A6 have shown dramatic changes in expression (Emberley, Murphy et al. 2004). S100A4 is probably the most characterised S100 protein in cancer and was originally named metastasin-1 due to its high expression in metastatic cell lines (Ebralidze, Tulchinsky et al. 1989; Ebralidze, Tul'chinskii et al. 1989). Elevated S100A4 levels in many forms of cancer

S100 Protein	Ortholog in mouse	Expression pattern (Normal)	Proposed Functions from literature	References
S100A1	Yes	Heart, skeletal muscle and brain	Involved in cardiac Ca^{2+} signalling	Most 2004, Treves 1997
S100A2	Yes	Some epithelial cells and keratinocytes	Regulates cytoskeletal interactions, tumour suppressor, chemoattractant for Eosinophils	Komada 1996, Gimona 1997, Wicki 1997
S100A3	Yes	Skin specific	None	
S100A4	Yes	Ubiquitous	Involved in cell migration, promotes metastisis, interacts with p53, myosin IIA and annexin II	Kim 2003, Garret 2005
S100A5	Yes	Kidney, Brain	Cu^{2+} homeostasis	Schafer 2000
S100A6	Yes	Ubiquitous	Regulates cell cycle, required for proliferation	Ferrari 1987, Breen 2003
S100A7	Yes	Skin, tongue	Chemoattractant for CD4 T-cells, interacts with cell survival mechanisms, antimicrobial activity	Jinquan 1996, Emberley 2005, Glaser 2005
S100A8	Yes	Myeloid cells, some epithelial cells	Inflammatory response, AA binding, chemo-attractant/repellent, antimicrobial, oxidative protection, development (see cross ref)	Lackman 1992, Harrison 1999, Sroussi 2006, Passey 1999

S100A9	Yes	Myeloid cells, some epithelial cells	Inflammatory response, AA binding, chemo-attractant/repellent, antimicrobial, oxidative protection (see cross ref)	Lackman 1992, Harrison 1999, Sroussi 2006
S100A10	Yes	Connective tissue, epithelia	Interacts with annexin II	Waismann 1995
S100A11	Yes	Haematopoietic cells, reproductive cells	Interacts with annexin I	Seemann 1996
S100A12	No	Lymphocytes, monocytes	Interacts with RAGE	Hoffmann 1999
S100A13	Yes	Heart and skeletal muscle	Involved in release of FGF-1 and IL-α, angiogenic role	Mouta Carreira 1998, Landriscina 2006, Sivaraja 2006
S100A14	Yes	Colon, thymus, kidney, liver and lung	Upregulated in cancer	Pietas 2002
S100A16	Yes	Unknown	Upregulated in cancer	Marenholz 2004
S100B	Yes	Brain	Neuronal Ca^{2+} signalling, Neurite growth and apoptosis	Huttunen 2000, Xiong 2000
S100P	Yes	Placenta	Associated with metastasis	Missiaglia 2004, Wang 2006
S100Z	Putative sequence	Leukocytes and spleen	Upregulated with other S100 proteins in Kawasaki disease	Ebihara 2005

Table 1.1 Putative functions of S100 family proteins. Adapted from references cited. Expression pattern taken from Swiss-Prot database.

are associated with poor prognosis and a metastatic phenotype (Rudland, Platt-Higgins et al. 2000). It is thought that S100A4 may inhibit the phosphorylation of p53 by protein kinase C (Sherbet and Lakshmi 1998; Grigorian, Andresen et al. 2001) preventing apoptotic cell death. It is not however thought that S100A4 plays a role in the initiation of cancer but rather in progression (Garrett, Varney et al. 2006) as overexpression of S100A4 does not cause cancer but can promote tumour progression once initiated (Davies, Rudland et al. 1996). S100A4 is believed to promote cancer cell motility through interactions with Myosin IIA (cytoskeletal remodelling) (Ford, Silver et al. 1997) and Annexin II (extracellular matrix remodelling) (Sherbet and Lakshmi 1998).

S100A7 is expressed in many tumour types and there are many suggested roles. S100A7 has been shown to regulate pro-survival NF-κB pathways through interaction with Jab-1 (Emberley, Niu et al. 2005) and is so strongly associated with the hyperproliferative condition psoriasis that it was originally named psoriasin (Madsen, Rasmussen et al. 1991). It is also thought that S100A7 could act in the response to cellular stress and anoikis, a form of apoptosis, to promote cell survival (Emberley, Niu et al. 2005). S100A6 is believed to play an as yet unknown role in regulating the cell cycle (Tonini, Casalaro et al. 1991; Breen and Tang 2003) and is found at elevated levels in breast, pancreatic and lung cancers. S100A2 is thought to act as a tumour suppressor and is down regulated in carcinomas due to gene silencing (Wicki, Franz et al. 1997). S100A2 expression is normal in primary tumours but down regulated in metastases (Boni, Burg et al. 1997; Boni, Heizmann et al. 1997). Forced re-expression of S100A2 in carcinoma cell lines had an effect that was the opposite of S100A4: inhibition of cell motility through cytoskeletal rearrangements (Nagy, Brenner et al. 2001). Rearrangement of chromosome 1q21 in humans is frequently observed in tumour cells (Ilg, Schafer et al. 1996) suggesting that S100 proteins could be involved in the development of cancer or

could be induced as a result of chromosome rearrangement as most are localised to this chromosome. The evidence so far seems to point to the former of those two possibilities. S100A8 and S100A9 are covered in section 1.2.

Consistent with its expression in the myocardium, S100A1 is associated with heart disease. Levels of S100A1 in the myocardium seem to correlate with heart performance as they are increased in hypertrophy (Ehlermann, Remppis et al. 2000) and lower during heart failure (Remppis, Greten et al. 1996). Elevated levels of S100B are found in patients with a variety of neurological conditions including Down's syndrome (von Eggeling, Freytag et al. 1993), Alzheimer's and multiple sclerosis (Schmidt 1998) although in Down's syndrome it is believed that trisomy 21 affects S100B levels as the gene is situated on chromosome 21. It is thought that normal levels of S100B in the brain promote neuron survival and growth whereas high extracellular levels caused by trauma have an apoptotic effect and can cause neurodegeneration (Rothermundt, Peters et al. 2003).

S100A7, S100A8, S100A9 and S100A12 are associated with inflammation. High serum levels of S100A8, S100A9 and S100A12 are associated with inflammatory diseases such as cystic fibrosis, rheumatoid arthritis and chronic bronchitis (Chilosi, Mombello et al. 1990; Roth, Teigelkamp et al. 1992), which is covered in more detail in section 1.3.

1.2.4 S100 mouse models

Knockout and transgenic mice have been generated for S100A1, S100B, S100A4, S100A8 and S100A9. All S100 null mice are viable and show no gross abnormalities except S100A8 null, which shows embryonic lethality

(Passey, Williams et al. 1999). More detail on the S100A9 and S100A8 null mice will follow in section 1.4.

S100A1 null mice show reduced response to beta-adrenergic stimulation consistent with reduced Ca^{2+} sensitivity. They also show reduced ability of the myocardium to compensate in a model of haemodynamic stress (Du, Cole et al. 2002). S100B null mice have been shown to display enhanced Ca^{2+} transients in cultured astrocytes (Xiong, O'Hanlon et al. 2000). However the cultures are prepared from 6-day S100B null mice and could not be seen in adult S100B null astrocytes *in vivo* (Nishiyama, Takemura et al. 2002). S100A4 null mice show decreased susceptibility to spontaneous tumour formation (Grum-Schwensen, Klingelhofer et al. 2005). Crosses of S100A4 null mice with spontaneous tumour forming mouse lines have shown that loss of S100A4 can reduce tumour progression and metastasis. However, transgenic mice, which over-express S100A4 in breast, show no increase in tumour formation (Garrett, Varney et al. 2006). This reinforces the idea that S100A4 is not involved in the initiation step in cancer but in tumour progression and metastasis. S100A11 null mice show no abnormalities and are viable and fertile. Even in Sertoli cells where S100A11 is highly expressed no defect can be found. One lesson that can be learned from the S100 null mice is that attributed *in vitro* functions are not always impaired when the protein is lacking. This suggests that either compensatory mechanisms apply or that many *in vitro* functions are not reproducible *in vivo*.

1.3 S100A8 and S100A9

1.3.1 S100A8/A9

These two S100 proteins were first identified in a number of separate investigations and given a correspondingly varied nomenclature. They were identified as the cystic fibrosis antigen, the human leukocyte antigen L1 (Dale, Fagerhol et al. 1983) heavy and light chains, migration inhibitory factor related proteins (MRPs) (Burmeister, Tarcsay et al. 1986) 8 and 14 (Hogg, Allen et al. 1989) and calprotectin (Steinbakk, Naess-Andresen et al. 1990). It was eventually found by cloning cDNA that this ensemble of antigens and proteins were in fact a heterodimer of two S100 proteins (Andersson, Sletten et al. 1988), which were named S100A8 and S100A9 in the reorganisation of S100 nomenclature (Schafer, Wicki et al. 1995). S100A8 is one of the smallest S100 proteins at 10.8kD whereas S100A9 is the largest S100 protein at 13.2kD.

S100 proteins typically form homodimers so the S100A8 and S100A9 heterodimer (S100A8/A9) was of interest. Immunoaffinity chromatography studies have shown that the 1:1 ratio of the heterodimer is the most favourable combination although higher order multimers and homodimers of S100A8 and S100A9 were found (Edgeworth, Gorman et al. 1991). A yeast 2-hybrid study confirmed the favoured status of the 1:1 heterodimer and showed that homodimers of the two proteins only occurred in the murine proteins (Propper, Huang et al. 1999). It has been shown that higher order multimers could exist *in vivo* (Teigelkamp, Bhardwaj et al. 1991). Homodimers of S100A8 and S100A9 have been shown to lack the complementary interface displayed by the heterodimer (Hunter and Chazin 1998). It should be noted that relatively few functions

have been proposed for homodimers of these S100 proteins whereas many functions have been proposed for the heterodimer.

1.3.2 Structure

S100A8 and S100A9 have been crystallised separately (Ishikawa, Nakagawa et al. 2000; Itou, Yao et al. 2002) and as a heterotetramer (Korndoerfer IP, Brueckner F et al. 2004) and they display structures typical of S100 proteins with an N-terminal "S100" EF hand and C-terminal "classical" EF hand as shown in fig 1.2. In S100A8 the C-terminal EF hand has an aspartic acid (Asp) in place of the S100 conserved glutamic acid (Glu). As Asp has a shorter side chain it was thought that this might impair Ca^{2+} binding. It has been shown, however, that a water molecule bridges the space between Asp 33 and Ca^{2+}.

S100A9 has an unusually long C-terminal extension making it the largest S100 protein. This C-terminus also shows low electron density during crystallisation suggesting it is flexible, which is consistent with a sequence rich in hydrophilic residues and no predicted secondary structure. Amino acids 89-108 in S100A9 show exact homology to Neutrophil Immobilising Factor (NIF-1) (Freemont, Hogg et al. 1989). This factor has been shown to inhibit the movement of neutrophils and monocytes but the relevance of this homology is in doubt as it has been shown that many of the residues on this section are involved in dimer formation and thus inaccessible (Itou, Yao et al. 2002). Amino acids 90-112 in S100A9 also show high homology with a sequence in high molecular weight kininogen (HMWK) (Hessian, Wilkinson et al. 1995).

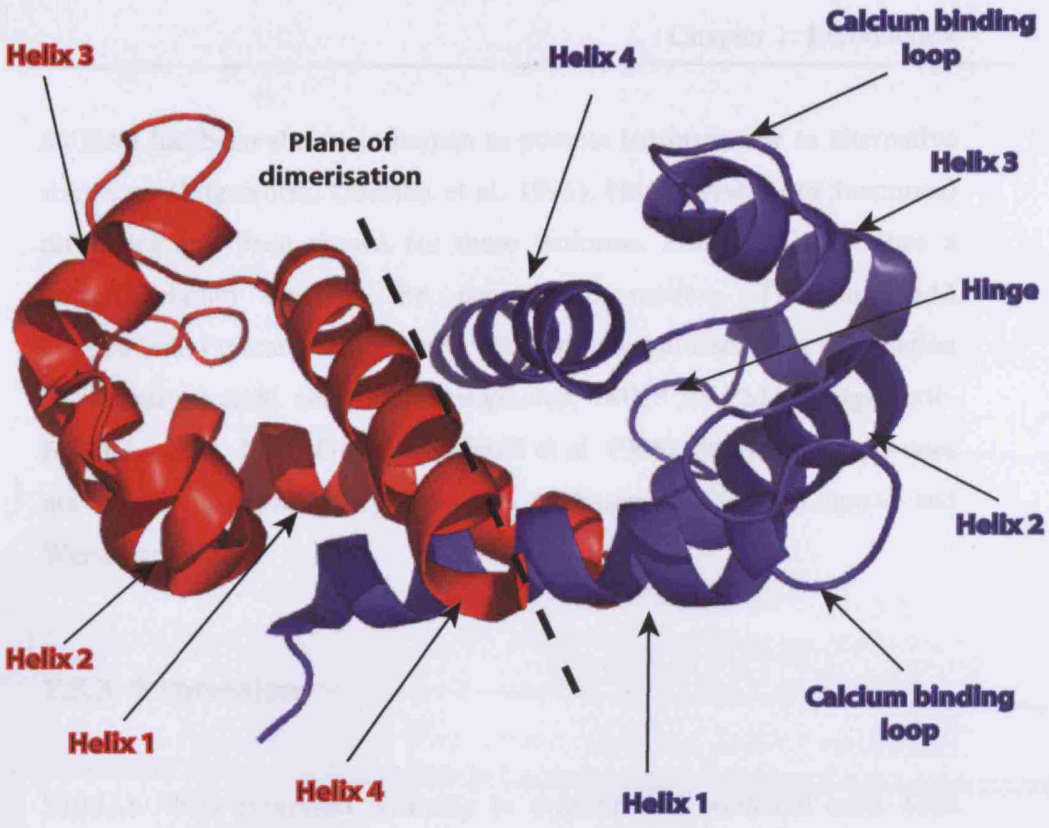


Fig 1.2 S100A8 and S100A9 protein heterodimer. Cartoon representation of S100A8 (Red) and S100A9 (Blue) heterodimer. Significant features are labelled. Figure adapted from structure entry: http://www.ncbi.nlm.nih.gov/Structure/mmdb/mmdbsrv.cgi?form=6&db=t&Dopt=s&uid=35490 with help from S.Griffiths, Molecular Enzymology Laboratory CRUK.

S100A9 has been shown in human to possess isoforms due to alternative start sites (Edgeworth, Gorman et al. 1991). No expression or functional difference has been shown for these isoforms. Human S100A9 has a phosphorylation site at the penultimate residue, Threonine 113 (Edgeworth, Freemont et al. 1989). Phosphorylation rises after stimulation of human myeloid cells with ionophores, fMLP or PMA (Edgeworth, Freemont et al. 1989; Guignard, Mauel et al. 1996). Murine S100A9 does not possess the phosphorylation site of human S100A9 (Lagasse and Weissman 1992).

1.3.3 Expression

S100A8/A9 is expressed normally in myeloid and epithelial cells. Like most S100 proteins cell type specific expression is seen and it is not ubiquitously expressed. In human S100A8/A9 comprises 45% of cytosolic neutrophil protein and in monocytes it comprises 1% of cytosolic protein (Edgeworth, Gorman et al. 1991). The expression seems dependant on differentiation status, as tissue macrophages do not normally express S100A8/A9 and murine monocytes have been shown to lose expression when they differentiate to macrophages (Lagasse and Weissman 1992; Goebeler, Roth et al. 1995). Immature myeloid cell lines such as HL-60 and U937 express low levels of S100A8/A9. When HL-60 cells are differentiated to monocytes however expression increases (Hogg, Allen et al. 1989). Also murine myeloid cells that express S100A8/A9 do so concurrently with Mac-1 and Gr-1 suggesting expression is confined to mature myeloid cells (Lagasse and Weissman 1992). The proteins are not expressed in other leukocytes, eosinophils or platelets.

A subset of normal squamous epithelia expresses S100A8/A9 including the tongue, oesophagus and cervix (Wilkinson, Busuttil et al. 1988). Some

epithelial cell lines also express the proteins and this is often linked to their differentiation ability. Cultured normal keratinocytes express the proteins whereas normal epidermis does not. Also expression is seen in the medulla of the hair shaft where expression overlaps with involucrin, suggesting terminally differentiated hair follicle cells (Saintigny, Schmidt et al. 1992; Thorey, Roth et al. 2001).

1.3.4 Expression in disease

Expression profiles for S100A8/A9 expand dramatically during certain disease conditions. The plasma of patients with cystic fibrosis shows elevated levels of S100A8/A9 (approximate 5-10 fold higher than control) (Roth, Teigelkamp et al. 1992). In conditions of chronic inflammation such as rheumatoid arthritis and pneumonia S100A8/A9 is expressed in macrophages as well as myeloid cells (Odink, Cerletti et al. 1987; Zwadlo, Bruggen et al. 1988). It is believed that these macrophages are derived from monocytes that did not shed their heterodimer expression upon differentiation. S100A8/A9 is also found in keratinocytes during psoriasis (Kelly, Jones et al. 1989), eczema and lupus (Wilkinson, Busuttil et al. 1988). Expression is largely in the spinous and granular layers of the epidermis although some basal expression can be seen. Squamous cell carcinomas also show high levels of the proteins (Schafer, Sachse et al. 1991).

S100A8/A9 expression is also upregulated in conditions of the epidermis like psoriasis and wound healing (Thorey, Roth et al. 2001; Broome, Ryan et al. 2003). It is thought that the proteins may play a role in stress-induced pathways and act to influence the immune response to these conditions (Eckert, Broome et al. 2004). A lot of the evidence in disease states seems to point towards association of S100A8/A9 with inflammation,

hyperproliferative conditions and rapid differentiation although no direct link has yet been conclusively proven.

Another condition associated with S100A8/A9 is a form of zincaemia where 5 patients (2 related) display infection, inflammation and metabolic problems (Sampson, Fagerhol et al. 2002). The patients have high plasma levels of S100A8/A9 (1.5-6.5 g/L compared with normal < 1mg/L) and it is thought that this may be chelating Zn^{2+} and causing the symptoms, which are consistent with low Zn^{2+}, although it is not known how the disease originates.

1.3.5 Cellular localisation

In resting myeloid cells S100A8/A9 is expressed in the cytosol (Edgeworth, Gorman et al. 1991). Stimulation with Ca^{2+} ionophores or zymosan has been shown to cause relocalisation to the membrane and also to intermediate filaments (van den Bos, Roth et al. 1996). Association with microtubules has been proposed as a potential mechanism of S100A8/A9 secretion (Rammes, Roth et al. 1997). It has been suggested that secretion of S100A8/A9 follows a novel path that is susceptible to microtubule disrupting agents. Recently it has been proposed that S100A8/S100A9 is found in primary and secondary granule fractions in neutrophils (Stroncek, Shankar et al. 2005) although this conflicts with previous reports of myeloid cell localisation (Edgeworth, Gorman et al. 1991). Several of the functions proposed for S100A8/A9 rely on secretion of the proteins and so any potential mechanism for this process would be of interest.

1.4 Proposed functions of S100A8/A9

1.4.1 Inflammation

The S100A8/A9 heterodimer has long been associated with inflammation and inflammatory conditions. It is highly expressed in immune cells like neutrophils, which are the first cells to arrive at a site of inflammation. It is also expressed in monocytes and has been shown in macrophages during acute and chronic inflammation (Odink, Cerletti et al. 1987; Zwadlo, Bruggen et al. 1988). Plasma levels of the proteins are raised in inflammatory disease and in Inflammatory Bowel Syndrome levels of S100A8/A9 in faeces are used as a non-invasive diagnostic. It is also believed that the proteins could have a role in monocyte activation (Hessian, Edgeworth et al. 1993). Activating stimuli cause S100A8/A9 in monocytes to relocate to the plasma membrane, cell surface and cytoskeleton (Lemarchand, Vaglio et al. 1992; Roth, Burwinkel et al. 1993; Burwinkel, Roth et al. 1994). The proteins have been located on the endothelium of venules near sites of inflammation (Hogg, Allen et al. 1989; Robinson, Tessier et al. 2002). This lead to the theory that S100A8/A9 could have a role in leukocyte trafficking and extravasation although no further evidence has confirmed this theory to date. A recent report has proposed that S100A8/A9 are involved in the inflammatory cascade during sepsis (Vogl, Tenbrock et al. 2007). The proposed model is S100A8 binding to the TLR-4/MD2 and inducing downstream TNF-α signalling.

It has been proposed that S100A8 and the S100A8/A9 heterodimer are chemotactic in both mouse and human. Murine S100A8 is proposed to attract both human and mouse neutrophils and mouse macrophages *in vitro* and *in vivo* (Lackmann, Cornish et al. 1992; Lackmann, Rajasekariah

et al. 1993; Devery, King et al. 1994). There is disagreement about whether the human proteins are chemotactic. One group shows human neutrophil chemotaxis in response to S100A8 and S100A8/A9, but the level at which they show the effect *in vitro* (10^{-12} M) is lower than the likely normal level *in vivo* (Roth, Vogl et al. 2003; Ryckman, Vandal et al. 2003). Also our group has shown that mice insensitive to endotoxin (C3H/HeJ) do not respond to recombinant S100A8/A9. Even when the group of Ryckman et al 2003 supplied the recombinant protein, the result could not be reproduced in the endotoxin insensitive mice. There is concern that chemotaxis could be induced by endotoxin present in recombinant samples, which seems the most likely conclusion. It should also be remembered that human and murine S100A8 show low homology (58%) for a cross species S100 protein comparison. This could suggest that human and murine S100A8 could have differing roles.

It has also been proposed that human S100A8 alone could be a chemo-repellent and anti-inflammatory agent. One study showed S100A8 to act as a repellent to neutrophils and that this effect could be blocked by oxidation of S100A8. Oxidation resistant mutants repelled neutrophils in an *in vivo* model in rat (Sroussi, Berline et al. 2006). This effect has recently been reproduced for S100A9 (Sroussi, Berline et al. 2007). Another study showed the S100A8/A9 complex reducing the concentration of inflammatory markers, IL-6 and nitric oxide, in an *in vivo* model of inflammation (Ikemoto, Murayama et al. 2007). This effect was reversed with injection of anti S100A8/A9 complex IgG. It is possible that if the two S100 proteins have a role in inflammation that this role could be dependent upon the inflammatory status and that the S100 proteins could act as either attractant or repellent as a secondary effect. No study has yet looked into this possibility and so it remains speculative.

Other S100 proteins have also been linked to inflammation with S100A7 thought to act as a chemoattractant. It is believed to recruit CD4 T cells and neutrophils but not CD8 T cells or monocytes. Despite the many links to inflammation no coherent picture has emerged as to the exact role the S100 proteins play *in vivo*, an impression that is confirmed by work in the null models.

1.4.2 Fatty acid binding

Arachidonic acid (AA) is a polyunsaturated fatty acid that has been shown to bind to the S100A8/A9 heterodimer with high affinity in a Ca^{2+} dependent manner (Kerkhoff, Klempt et al. 1999). Monomers and homodimers of either S100A8 or S100A9 do not bind AA. The interaction is inhibited by other unsaturated fatty acids but not by saturated ones (Kerkhoff, Klempt et al. 1999). Additionally HL-60 cells stimulated with Ca^{2+}ionophores or phorbol ester release both S100A8/A9 and AA. It has been proposed that S100A8/A9 binding of AA is part of a trans-cellular process of leukotriene synthesis (Kannan 2003). It is believed that S100A8/A9 is responsible for Ca^{2+} mediated AA transport from the cell. This leads to leukotriene synthesis and neutrophil degranulation in an extracellular nucleotide triggered mechanism (Kannan 03). This process is believed to exacerbate inflammation and correlates with the high S100A8/A9 expression at inflammatory sites. It is believed that S100A8/A9 is the major fatty acid binding complex in neutrophils, which would provide a role for S100A8/A9 in that cell type and explain the high levels of expression.

1.4.3 Antimicrobial

One of the original names for the S100A8/A9 complex was calprotectin, reflecting evidence that it had antimicrobial properties. At low concentrations (4-128 μg/ml) it inhibits the growth of Candida species and *Cryptococcus.neoformans.* At higher concentrations (64-256 μg/ml) it inhibits the growth of bacteria such as *Escherichia.coli*, *Staphylococcus.aureus*, and *Klebsiela* (Santhanagopalan 95). The antimicrobial effect is independent of Ca^{2+} yet is dependent on Zn^{2+}. Candida grown in Zn^{2+} enriched conditions proved resistant to S100A8/A9 growth inhibition. Polyhistidine, a Zn^{2+} chelator, also inhibited microbial growth in a Zn^{2+} dependent fashion. Microbes require Zn^{2+} for vital enzymes such as DNA and RNA polymerases and S100A8/A9 may exert its anti-microbial activity by chelating Zn^{2+}. It has been shown that S100A8/A9 levels in abscess fluid are higher than the level required for microbe inhibition suggesting this may indeed be an *in vivo* role (Sohnle, Collins-Lech et al. 1991). As many of the functions proposed for S100A8/A9 occur in areas of inflammation a secondary characteristic of anti-microbial activity would definitely prove beneficial.

1.4.4 Protection from oxidative damage

It has been proposed that S100A8, when oxidised can form homodimers linked by disulphide bonds. It is suggested that S100A8 oxidation could be a protection mechanism preventing reactive oxygen species from damaging tissues (Harrison, Raftery et al. 1999). The evidence is that exposure to hypochlorite causes S100A8 homodimers to appear which are 92 Da heavier than expected. These homodimers have been seen both *in vitro* and in lung lavage fluid from endotoxin-induced pulmonary injury (Harrison, Raftery et al. 1999).

1.4.5 Other functions

The C terminal domain of S100A9 shows homology to high molecular weight kininogen (HMWK), which is capable of interacting with anionic surfaces in the blood clotting process. S100A9 and also S100A9 complexed with S100A8 caused delayed onset of plasma coagulation in *in vitro* studies (Hessian, Wilkinson et al. 1995). It has been proposed that the A8/A9 complex may have a role preventing fibrin formation at sites of leukocyte migration.

It has also been proposed that A8/A9 is an inducer of cytostasis and apoptosis. The complex has been shown to inhibit growth of leukocytes, bone marrow cells and certain cell lines. It has been shown to induce apoptosis in normal fibroblasts and a human leukaemia cell line. However this effect is reversed by Zn^{2+} showing that the effect may be similar to the antimicrobial effect proposed for the A8/A9 complex (Yui, Mikami et al. 1995; Yui, Mikami et al. 1995; Yui, Mikami et al. 1997).

S100A8/A9 has been shown to enhance activation of NADPH oxidase in neutrophils. It is thought to associate with a subunit called p67phox and Rac via S100A8. It is believed to both activate NADPH oxidase and deliver arachidonic acid to the complex, which could be the activating agent (Doussiere, Bouzidi et al. 1999; Doussiere, Bouzidi et al. 2002; Kerkhoff, Nacken et al. 2005). Impaired oxidase activity has been shown with antibody-mediated blockage of S100A8/A9 and with mutants lacking AA binding sites (Kerkhoff, Nacken et al. 2005).

Many of the proposed *in vitro* functions for S100A8/A9 derive from *in vitro* studies of the proteins. Some of the *in vitro* data is directly

contradictory, while the sum of proposed roles shows a protein complex with an astonishingly wide array of functions. As can be seen in the next section many *in vitro* results were not reproducible within *in vivo* model. It is always desirable to examine the *in vivo* models when assessing S100A8/A9 function.

1.4.6 S100A9 null mice

Our group and another have produced and characterised S100A9 null mice (Hobbs, May et al. 2003; Manitz, Horst et al. 2003). Both groups found that the S100A9 null mouse is viable and shows no gross abnormality. Despite its high expression in myeloid cells S100A9 seems to be redundant in myelopoiesis. Many aspects of S100A9 null neutrophil function have been tested including phagocytosis, superoxide burst and apoptosis and have been found to be normal (Hobbs, May et al. 2003).

An interesting finding by both groups is that in myeloid cells the loss of S100A9 also caused loss of S100A8 protein but not mRNA as shown in fig 1.3. It seems that S100A8 protein, in myeloid cells at least, is not stable without its heterodimer partner, S100A9. In neutrophils, where the heterodimer comprises 45% of cytosolic protein, this results in decreased neutrophils density on a Percoll gradient (Hobbs, May et al. 2003). No S100 protein family member is upregulated and on a 2D gel of neutrophil lysate no other protein is upregulated in S100A9 null neutrophils compared to wildtype. It was thought that the loss of such an abundant protein complex would cause severe problems and/or engage compensatory mechanisms. The lack of such a response was very surprising. The groups disagree about S100A8 expression in bone marrow as S100A8 expression was seen in S100A9 null bone marrow in 70% fewer cells compared to wildtype using cytospin and immunostaining

Fig 1.3 Analysis of S100A8 and S100A9 mRNA and protein expression in wildtype and S100A9 null bone marrow lysates. Expression of S100A8 and S100A9 mRNA in bone marrow cells from wildtype and S100A9 null mice (A) is shown. Expression of S100A8 and S100A9 protein from the same bone marrow samples is shown in western blot (B) and in 2D western blot. Reproduced from Hobbs et al 2003.

(Manitz, Horst et al. 2003). Our group could not repeat this result either by immunostaining or by western blot or FACS staining.

Ca^{2+} responses to inflammatory agents like MIP-2 are altered in S100A9 null neutrophils. The defect lies in the IP3 mediated Ca^{2+} response and occurs at the level of PLC-β (McNeill, Conway et al. 2007), yet despite this evidence S100A9 null neutrophils can still respond to MIP-2. *In vitro* and *in vivo* models of peritonitis show that S100A9 null mice show no difference in neutrophil recruitment. One group also reports a defect in

S100A9 null neutrophil activation. Stimulation of S100A9 null neutrophils with IL-8 lead to a reduced CD11b upregulation when compared with wildtype (Manitz, Horst et al. 2003). It was also seen that S100A9 null neutrophils did not migrate in a 3D collagen matrix migration assay. Our group could not repeat these results. The differences in the two strains of S100A9 null mice cannot so far be explained but could occur due to a fundamental difference in the mice due to strain (both groups used C57BL/6J mice) or due to experimental design and interpretation. Our S100A9 null mice also show greater susceptibility to papilloma formation in a skin carcinogenesis protocol although at present it is unknown whether this is a function of neutrophil activity or of keratinocyte function (E.McNeill - unpublished) as both are known to express S100A9 and S100A8 in this model. Recently is has been shown that S100A9 null mice are more resistant to models of LPS induced sepsis (Vogl, Tenbrock et al. 2007). The S100A9 null mouse has shown that, although many functions can be ascribed to a protein *in vitro*, *in vivo* data are of vital importance. The large number of attributed *in vitro* functions, which are not impaired by an *in vivo* knockout, is striking. This shows that the function of S100A8/A9 is difficult to approach from *in vitro* studies.

1.4.7 S100A8 null mice

S100A8 null mice are embryonic lethal. It has been proposed that the embryonic lethality occurs between E 8.5 and E 9.5 (Passey, Williams et al. 1999) with null embryos being resorbed by E 13.5.

It was thought that the earliest expression of S100A8 in murine haematopoietic development occurred in the liver at E 10.5. This has been shown to be in conjunction with S100A9 in early myeloid cells (Lagasse and Weissman 1992). It was then claimed that S100A8 mRNA was expressed within a subset of cells around the ectoplacental cone at E 6.5 to E 7.5. It was proposed that this population is responsible for mediating the maternal foetal interactions directly after implantation (Passey, Williams et al. 1999). The justification for this is that S100A8 is believed to be involved in inflammation and implantation can be seen as a form of acute inflammation (Brandon 1993; McMaster, Dey et al. 1993). Another theory is that S100A8 can protect the embryo against reactive oxygen species, as it can be rapidly oxidised (Harrison, Raftery et al. 1999).

However, there were distinct problems within the study of the S100A8 null mouse. Data was not shown indicating the protein expression of S100A8. As the S100A9 null mouse has demonstrated, in some cases S100A8 mRNA can be expressed with no protein subsequently produced. The interpretation of S100A8 mRNA labelling is also controversial and has been stated to be of foetal origin (Passey, Williams et al. 1999) or of maternal origin (Hobbs, May et al. 2003). This issue is of vital importance as foetal expression can explain an embryonic lethal phenotype whereas maternal expression, on its own, cannot. It is also unclear that if S100A8 is responsible for establishing implantation of the embryo why the lethality occurs much later in development at E 9.0.

1.4.8 Is there a role for S100A8 independent of S100A9?

One issue that arose with the S100A8 and S100A9 null mice is that the two proteins were believed to be co-expressed based on evidence from myeloid cells and disease conditions. The S100A9 null is viable but shows loss of S100A8 in myeloid cells. The loss of S100A8 has been shown to be embryonic lethal. For the two observations to be reconciled S100A8 must be stably expressed independently of S100A9 in development. If this were not the case then the S100A9 mouse should also be embryonic lethal.

1.5 Preimplantation development

Development from a fertilised 1-cell zygote through to birth in the mouse is an immensely complex process and is briefly summarised in fig 1.4. In summary an embryo divides and differentiates rapidly before implantation, whereupon it continues to develop and further differentiate *in utero* until birth as summarised in fig 1.4. Advances in developmental biology make it possible to understand many of the molecular events of this process, but the picture is far from complete. For convenience the events of development will be subdivided into preimplantation and postimplantation. Furthermore emphasis is placed on aspects of development more relevant to this thesis as is illustrated in overview form. Preimplantation development explains how fertilisation occurs and how the 1-cell zygote divides and differentiates ready to implant.

1.5.1 Spermatogenesis

Spermatogenesis is the process of male gamete production and involves cell division and differentiation from diploid spermatogonia to haploid sperm cells as shown in fig 1.5 (Brinster 2007). It takes place within the seminiferous tubules of the testes and involves mitotic proliferation of spermatogonia, which form a pool of spermatogenic stem cells with self-renewal ability (Brinster 2002). Migration of spermatogonia through spermatogenic supporting Sertoli cells is necessary to further differentiate. Meiotic cell division occurs at the primary and secondary spermatocyte stages to produce haploid spermatids. Finally differentiation from spermatids to sperm cells involves gross morphogenic changes from a rounded cell to a structured and functional sperm cell. Cross sections of the seminiferous tubules expose all sperm differentiation stages with the

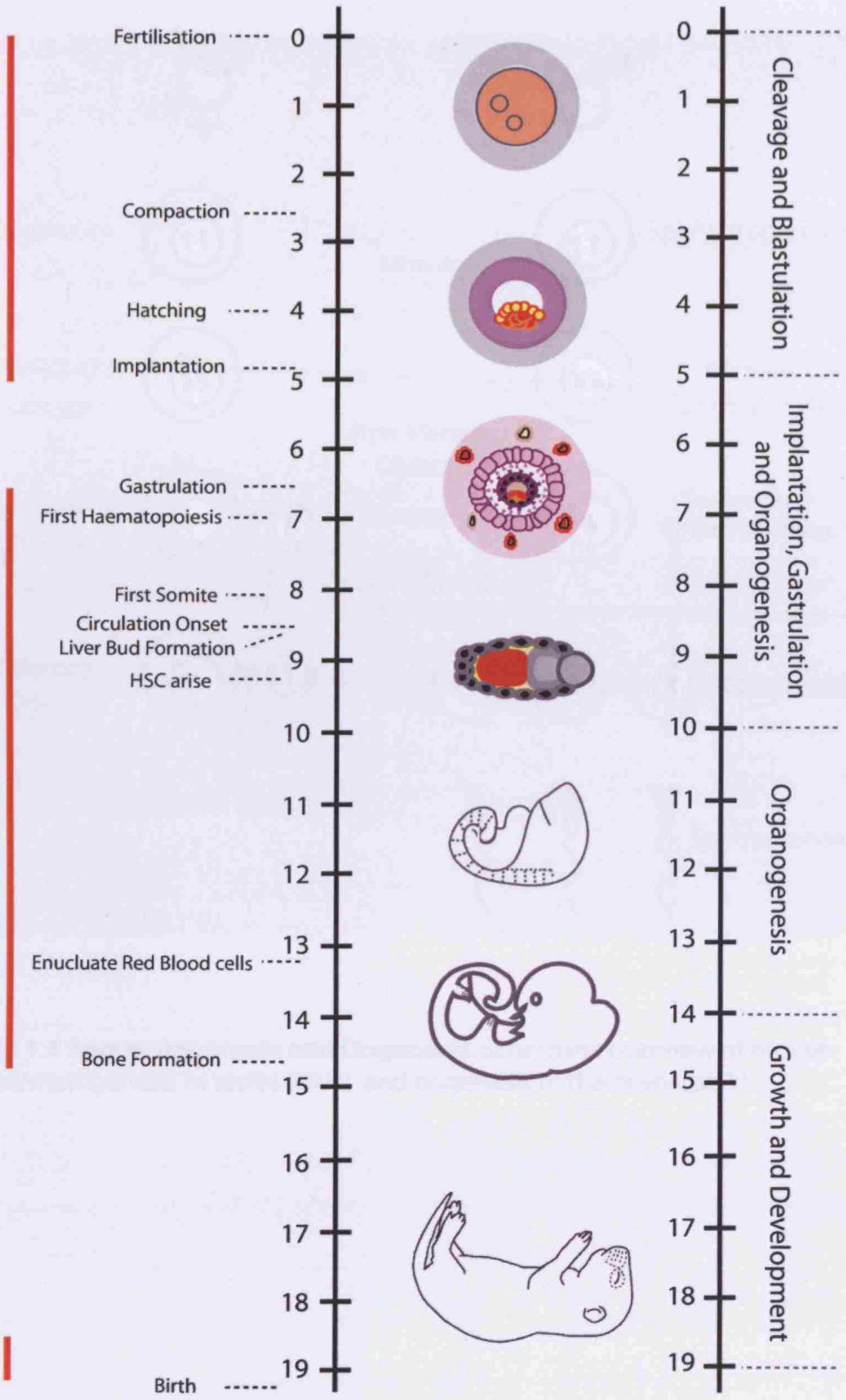


Fig 1.4 General overview of mouse development. Significant events in mouse development with areas under study in this thesis highlighted (Red).

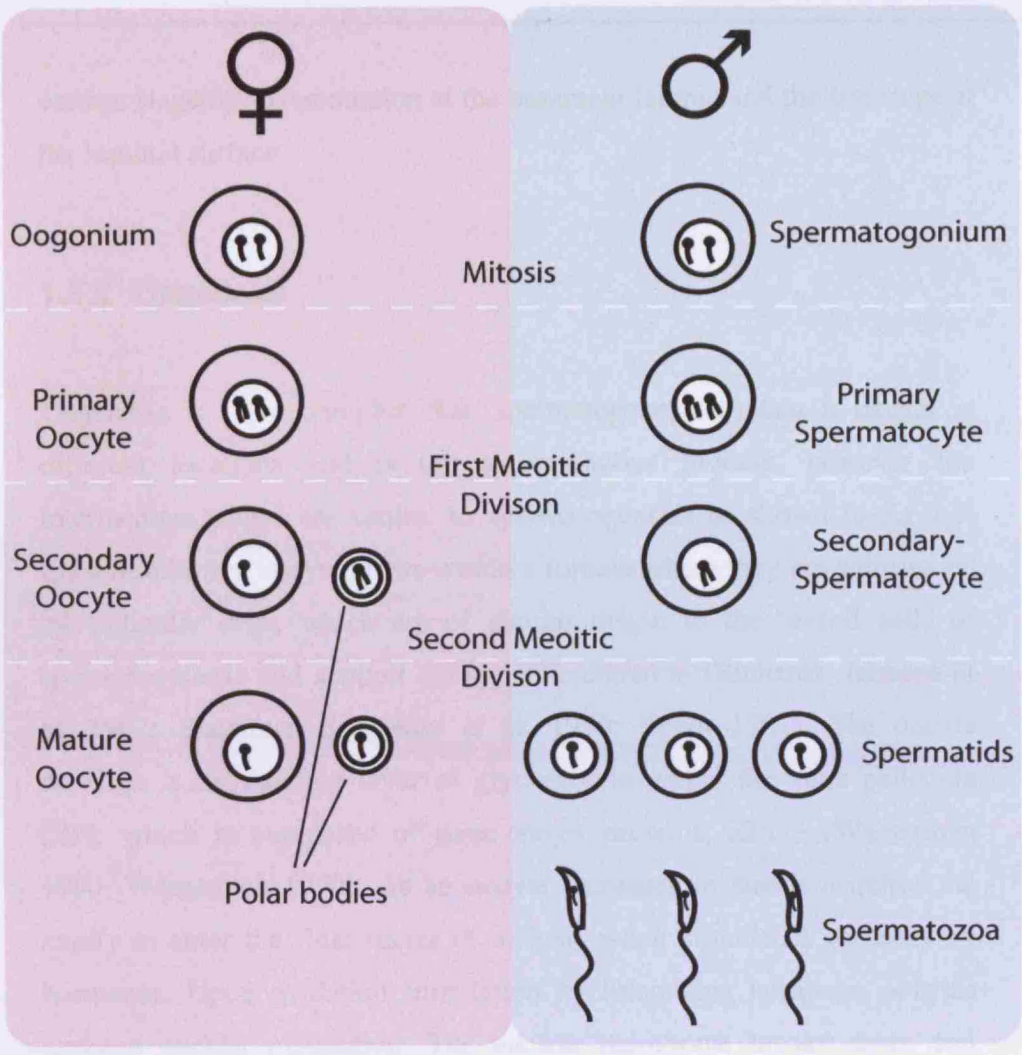


Fig 1.5 Spermatogenesis and Oogenesis. Schematic overview of mouse spermatogenesis in testes (blue) and oogenesis in the ovary (pink).

earliest stage of differentiation at the basement lamina and the last stage at the luminal surface.

1.5.2 Oogenesis

Oogenesis is more complex than spermatogenesis in that it occurs in different locations and is not a continuous process, however the intermediate stages are similar to spermatogenesis as shown in fig 1.5. Each developing oocyte grows within a follicle where they are surrounded by follicular cells, which are of similar origin to the Sertoli cells of spermatogenesis and support oocyte differentiation (Richards, Jahnsen et al. 1987; Buccione, Schroeder et al. 1990; Eppig 1991). The oocyte develops a surrounding layer of glycoprotein called the zona pellucida (ZP), which is composed of three major proteins, ZP1-3 (Wassarman 1990; Wassarman 1990). As an oocyte increases in size it acquires the ability to enter the final stages of meiosis when stimulated correctly by hormones. Upon ovulation stimulation by luteinising hormone, oocytes undergo nuclear maturation. The nuclear membrane breaks down and chromosomes assemble on the spindle and polarise within the cell. One set of chromosomes is extruded with some cytoplasm as the first polar body. The other set of chromosomes remains in meiotic arrest and the oocyte is released from its follicle as a secondary oocyte. Oocytes remain in meiotic arrest until after fertilisation. Fertilisation triggers the second round of meiosis, which produces a second polar body to form the fully mature oocyte.

1.5.3 Fertilisation

Fertilisation is the process of 2 gametes, oocyte and sperm, joining together to form a zygote. It comprises distinctly ordered steps starting with sperm and oocyte contact. There is evidence that sperm are drawn to the oocyte by chemo-attractants produced by oocyte follicular cells (Eisenbach 1999; Eisenbach 1999; Eisenbach and Tur-Kaspa 1999). The first part of the process begins when a sperm with an intact acrosome binds to the glycoprotein membrane surrounding the oocyte, which is called the zona pellucida (ZP) (Bleil and Wassarman 1983). This process is highly species specific in mammals and is mediated by receptors on both sperm and oocyte. In mice oocyte ZP3 is known to act as a sperm receptor via linked oligosaccharides (Wassarman 1990; Rosiere and Wassarman 1992) and is thought to interact with proteins like SED1 (Ensslin and Shur 2003) and CD9 (Kaji, Oda et al. 2002) on sperm.

Once bound to the ZP the sperm prepares to penetrate by undergoing the acrosomal reaction (Abou-Haila and Tulsiani 2000). The acrosome is a large lysosome derived from golgi and situated at the apical region of the sperm head. The acrosome reaction is similar to exocytosis and is initiated by sperm fusion with the ZP. The reaction causes the sperm plasma membrane and acrosomal membranes to fuse and release acrosomal contents to degrade the ZP (Abou-Haila and Tulsiani 2000). The acrosomal reaction makes it possible for the sperm to penetrate the ZP by a combination of sperm motility and acrosomal enzyme activity breaking down the ZP. The sperm then enters the perivitelline space. The penetrating sperm can then bind to the plasma membrane of the oocyte. After binding the sperm and oocyte membranes fuse. Fusion of a single sperm prevents the oocyte plasma membrane fusing again and should prevent other sperm from penetrating the ZP. At this point the oocyte has

been fertilized with the entire process occurring in approx 90 mins for mice. Fertilisation triggers a second round of meiosis in the oocyte as discussed above. Nuclear membranes form round the sperm and oocyte chromosomes forming pronuclei (Maro, Howlett et al. 1986; Maro, Johnson et al. 1986; Poccia and Collas 1996). The pronuclei each replicate their DNA before they meet in the centre of the oocyte (Maro, Howlett et al. 1986; Maro, Johnson et al. 1986). Binding of the pronuclei and dissolution of their membranes allows for both sets of chromosomes to assemble on a common spindle. The first mitotic cleavage to a 2-cell zygote occurs shortly after this process.

1.5.4 Expression of the zygotic genome

Early development requires dynamic cell division soon after fertilisation and many macromolecules are required. In order to accomplish this the oocyte is provided with RNA and protein to drive the early processes successfully (Schultz and Heyner 1992). Integration of sperm and oocyte genetic material and expression of zygotic RNAs and protein is essential for further development and so a switch must be made. The process of going from exclusively maternal to zygotic gene products is called the maternal-zygotic transition. Degradation of maternal transcripts occurs (Paynton, Rempel et al. 1988) and translation of maternal transcripts is severely reduced. This transition is not an instantaneous event and not all maternal proteins are replaced. Some maternal proteins can remain active for many days, even up until implantation (Schultz and Heyner 1992). In mice the transition occurs at the 2-cell stage of development (Flach, Johnson et al. 1982). The zygotic genome is deliberately silenced during early development (Wiekowski, Miranda et al. 1991) and the transition mainly consists of de-repression of the zygotic genome, de-novo embryonic transcription and degradation of maternal transcripts. When

approaching early development with gene silencing in mind it is important to bear in mind the early reliance of the oocyte, 1 and 2-cell stages on maternal transcripts and protein for function, as homozygous null embryos often have delayed exhibition of null phenotypes.

1.5.5 Cell division

With such a long way to go from 1 cell to mouse embryo, division in early development is rapid and must set the stage for the differentiation events required to produce a blastocyst ready for implantation as shown in fig 1.6. The zygote divides from 1 cell to 2 and subsequently 4 and 8 cells. It was thought that in mice cell division creates equally potent blastomeres until the 8-cell stage of development (Tarkowski and Wroblewska 1967; Johnson and McConnell 2004) although current evidence has suggested that cell fate can be determined as early as the first division (Piotrowska, Wianny et al. 2001). Cell division at these stages involves increase in the number of cells with negligible increase in embryo size. The building blocks for the created cells all exist in the oocyte prior to fertilisation and the embryo does not significantly increase in size despite multiple cell divisions. Studies have shown that the cell lineage initiation events have their origin in early cell division before morphogenic changes make lineage differentiation clear beginning at the 8-cell stage.

1.5.6 Compaction

Compaction occurs at the late 8-cell stage and is the first noticeable change of shape and structure in the preimplantation embryo. It is characterised by a change from loosely associated blastomeres to increasingly tight cell-cell interactions (McLachlin, Caveney et al. 1983). Blastomeres become impossible to distinguish and the embryo takes on a

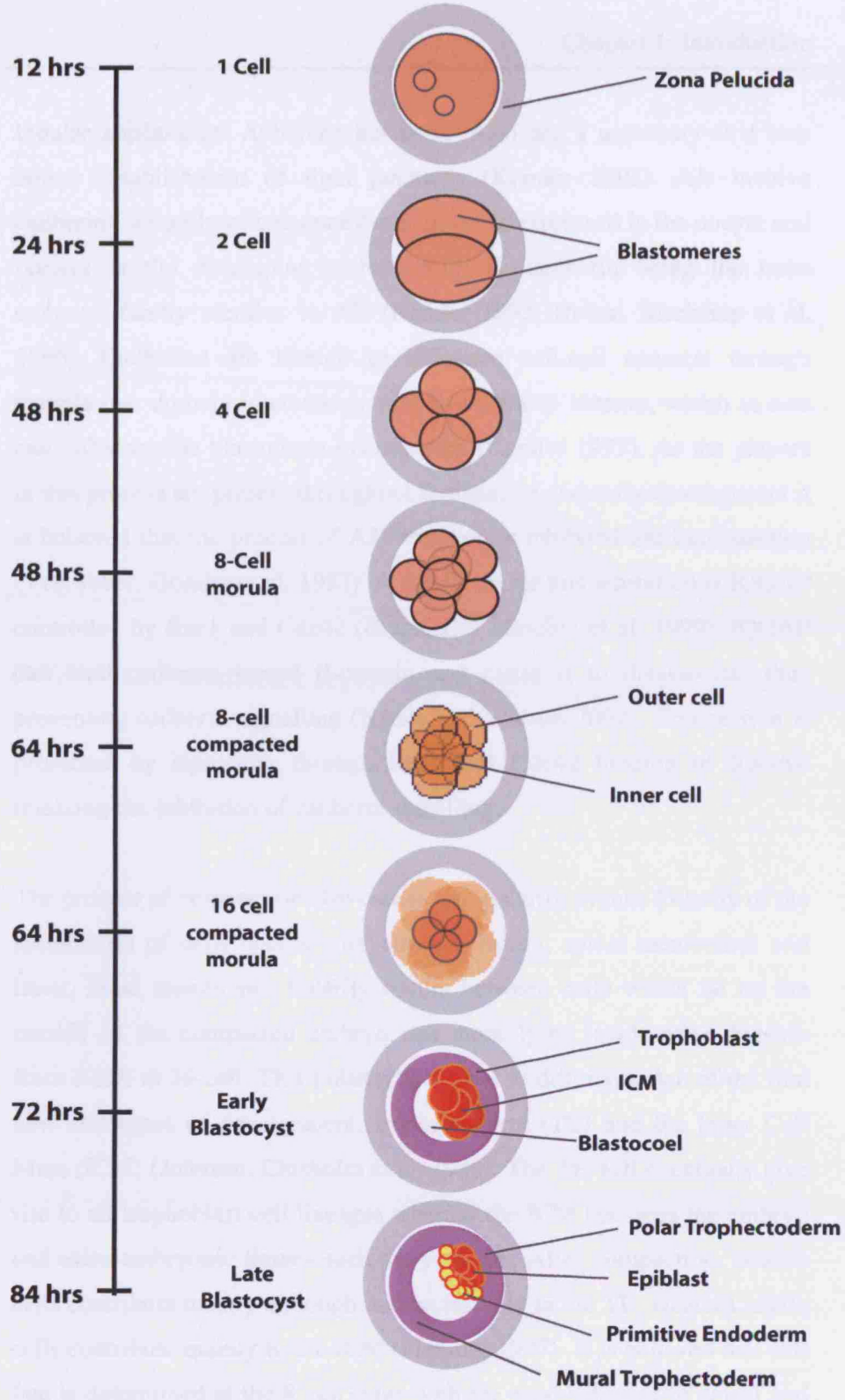


Fig 1.6 Progression of development prior to implantation. Development from 1 cell to pre implantation blastocyst showing the cell division processes with undifferentiated blastomeres (Orange), first differentiated cell types (Trophoblast -purple, Inner cell mass - Red) along with further differentiation (Epiblast -Red, primitive endoderm - Yellow).

lobular appearance. Adherens junctions (AJs) are a necessary first step before establishment of tight junctions (Kemler 1993). AJs involve cadherins, a family of transmembrane proteins expressed in the oocyte and present in the developing embryo with E- cadherin being the most common family member in AJs (Kemler 1993; Huber, Bierkamp et al. 1996). Cadherins are though to influence cell-cell contacts through cytoplasmic domain interactions with β- and/or γ- catenin, which in turn can influence the blastomere cytoskeleton (Kemler 1993). As the players in this process are present throughout fertilisation and early development it is believed that the process of AJ formation is inhibited until compaction (Vestweber, Gossler et al. 1987). A candidate for this inhibition is IQGAP controlled by Rac1 and Cdc42 (Braga, Del Maschio et al. 1999). IQGAP can bind cadherin bound β-catenin and cause it to disassociate thus preventing cadherin signalling (Natale and Watson 2002). Compaction is promoted by signalling through Rac1 and Cdc42 binding of IQGAP releasing the inhibition of cadherin signalling.

The process of compaction also causes two polarity events. Polarity of the membranes of cells occurs with outward facing, apical membranes and inner, basal membranes Polarity occurs between cells which lie on the outside of the compacted embryo and those lying inside after division from 8-cell to 16-cell. This polarity is crucial to differentiation of the first new cell types of development, trophectoderm (TE) and the Inner Cell Mass (ICM) (Johnson, Chisholm et al. 1986). The TE will eventually give rise to all trophoblast cell lineages whereas the ICM becomes the embryo and extra-embryonic tissues such the yolk sac. After compaction, outside cells contribute mainly although not exclusively to the TE, whereas inside cells contribute mainly to the ICM (Fleming 1987). It is believed that cell fate is determined at the 8 cell stage with the establishment of apical and basement membranes, which are characterised by differential molecular markers. The apical membrane is characterised by Jam-1, Ezrin, PKCs and

Par proteins D3 and D6b (Pauken and Capco 2000; Thomas, Sheth et al. 2004; Vinot, Le et al. 2005). Basement membranes are characterised by Par 1, Epithin, E-cadherin and β-catenin (Vestweber, Gossler et al. 1987; Vinot, Le et al. 2005). Subsequent cell divisions give rise to TE or ICM cells determined by the plane of division (Johnson and Ziomek 1981; Johnson and Ziomek 1981). Symmetrical division gives rise to two TE fate cells each of which inherit the apical and basal domains. Asymmetric division gives rise to a TE fate cell, which inherits the apical membrane and an ICM fate cell inheriting the basal membrane (Sutherland, Speed et al. 1990).

1.5.7 Cavitation

Cavitation is the process that converts a compacted morula to a blastocyst. It involves formation of a liquid filled cavity in the centre of the embryo, called the blastocoel, and the tightening of junctions and flattening of TE cells (Biggers, Borland et al. 1977). The cavity is formed due to a combination of the TE tight junctions with water and ion transport systems. It was thought that Na+ K+-ATPase is a main regulator of cavity formation by promoting ionic gradients across the TE (Watson and Barcroft 2001). An alternative explanation involving water channels called Aquaporins (AQPs) has been proposed (Offenberg, Barcroft et al. 2000). Diffusion is the most efficient method of moving water across the TE but it has not been shown that the necessary gradients across the TE exist. AQPs can transport water across the TE at near iso-osmolar levels (Deen and van Os 1998). Functional AQPs have been shown to be present in TE membranes (Barcroft, Offenberg et al. 2003). Tight junction formation is essential in forming the blastocoel as it prevents free diffusion and the collapse of gradients.

1.5.8 Blastulation

The blastocyst once formed is itself polarised with TE forming a flattened outer cell layer and ICM concentrated at one pole. This polarisation is reflected in the TE with the trophoblasts at the same pole as the ICM becoming the polar trophectoderm and those surrounding the blastocoel becoming the mural trophectoderm. 24 hours after blastocyst formation a further lineage event occurs with the ICM segregating to form the epiblast (EPI - future embryo) and primitive endoderm (PE - future yolk sac) (Gardner and Rossant 1979). The PE forms a monolayer on the ICM facing the blastocoel. It was originally believed that the establishment of the EPI and PE lineages was determined by cell position within the ICM with the cells facing the blastocoel becoming PE and the inner, enclosed ICM cells becoming EPI (Martin and Evans 1975). However current evidence points to a model of ICM heterogeneity where the two cell types exist within the ICM and the two lineages are sorted by migration to form the structure of EPI and PE (Chisholm and Houliston 1987; Chazaud, Yamanaka et al. 2006).

1.5.9 Hatching

In order to prepare for implantation a blastocyst must shed its outer zona pellucida in a process called hatching. The zona is thought necessary not for the development of the blastocyst but in preventing premature adhesion of the developing blastocyst. At approx E 5.0 the blastocyst hatches from the surrounding zona pellucida using a combination of physical and enzymatic action. Enzymes from the mural trophectoderm and maternal uterus are believed to digest the ZP matrix (Perona and Wassarman 1986; Sharma, Liu et al. 2006). Hatching is also facilitated by rhythmic contraction and expansion of the blastocyst, which helps rupture

the weakened ZP (Niimura 2003). The embryo emerges lead by the mural trophectoderm.

1.5.10 Preimplantation lethal phenotypes

There are a number of common preimplantation lethal phenotypes and it is always possible that a new phenotype may be displayed when studying a new lethal phenotype. Increasingly work with RNAi and morpholinos is extending the range of preimplantation phenotypes. It is possible that no mutant embryos are generated, which is caused by a haploid effect on sperm or oocytes (Gliki, Ebnet et al. 2004). Mutant embryos may delay or arrest during early cell division processes ultimately causing lethality. Compaction could be defective with failure of cell adhesion being a common cause of lethality at this stage (Riethmacher, Brinkmann et al. 1995; Natale, Paliga et al. 2004). Failure to cavitate can be caused by arrest or a failure to form and maintain a fluid import across the trophoblast layer (Thomas, Sheth et al. 2004). Abnormal blastocyst morphology can occur such as loss of tight junction integrity or lack of an ICM due to defective cell signals (Nichols, Zevnik et al. 1998). Excessive cell death at this stage can cause problems forming a functional blastocyst. Failure to hatch from the zona pellucida is typically caused by defects in the trophectoderm and is common in wildtype (approx 20%) and could be completely prevented in a mutant embryo. Preimplantation development is extremely complex and involves many processes, any of which can potentially go wrong and cause embryo lethality.

1.5.11 Implantation

If development proceeds successfully to the formation of blastocyst the next step is implantation. The zona pellucida is shed and the embryo must

establish an interface with the maternal endometrium. Implantation is necessary to allow an embryo to fuel its accelerating development by accessing the maternal blood supply and its nutrients. Three steps occur in implantation; apposition is where trophoblast cells are apposed to the luminal epithelium of the uterus, adhesion where trophoblast and luminal epithelial become attached strongly enough to resist luminal flushing and invasion where trophoblast cells invade the uterine epithelium resulting in loss of the maternal epithelium by apoptosis (Schlafke and Enders 1967; Enders and Schlafke 1969) as shown in fig 1.7. The process is continuous and involves complex mechanisms of hormonal control, adhesion, tissue remodelling and of course an array of signalling events to co-ordinate the process (Makrigiannakis and Minas 2007). Implantation is eccentric in mice, which means that the maternal decidua forms an invagination upon embryo attachment.

Apposition involves uterine swelling or oedema, which closes the uterine lumen. This has been shown to happen in response to ovarian steroids and will occur even in the absence of blastocysts. The receptivity of the uterus is highly important in implantation and is largely controlled by the ovarian steroids progesterone and oestrogen. The actions of oestrogen and progesterone co-ordinate the proliferation and differentiation of the uterine cells especially the epithelium (Huet-Hudson, Andrews et al. 1989). In mice oestrogen is crucial in promoting receptivity of the endometrium but less essential in subsequent implantation events. Trophectoderm and the luminal epithelium become apposed before the attachment reaction occurs. The uterine lumen closes due to the action of progesterone (Finn and Martin 1976), which brings uterine luminal epithelium and blastocyst trophoblast in close proximity. Vascular permeability increases significantly at sites of apposition, which can be shown by molecular dye penetration (Ljungkvist and Nilsson 1974).

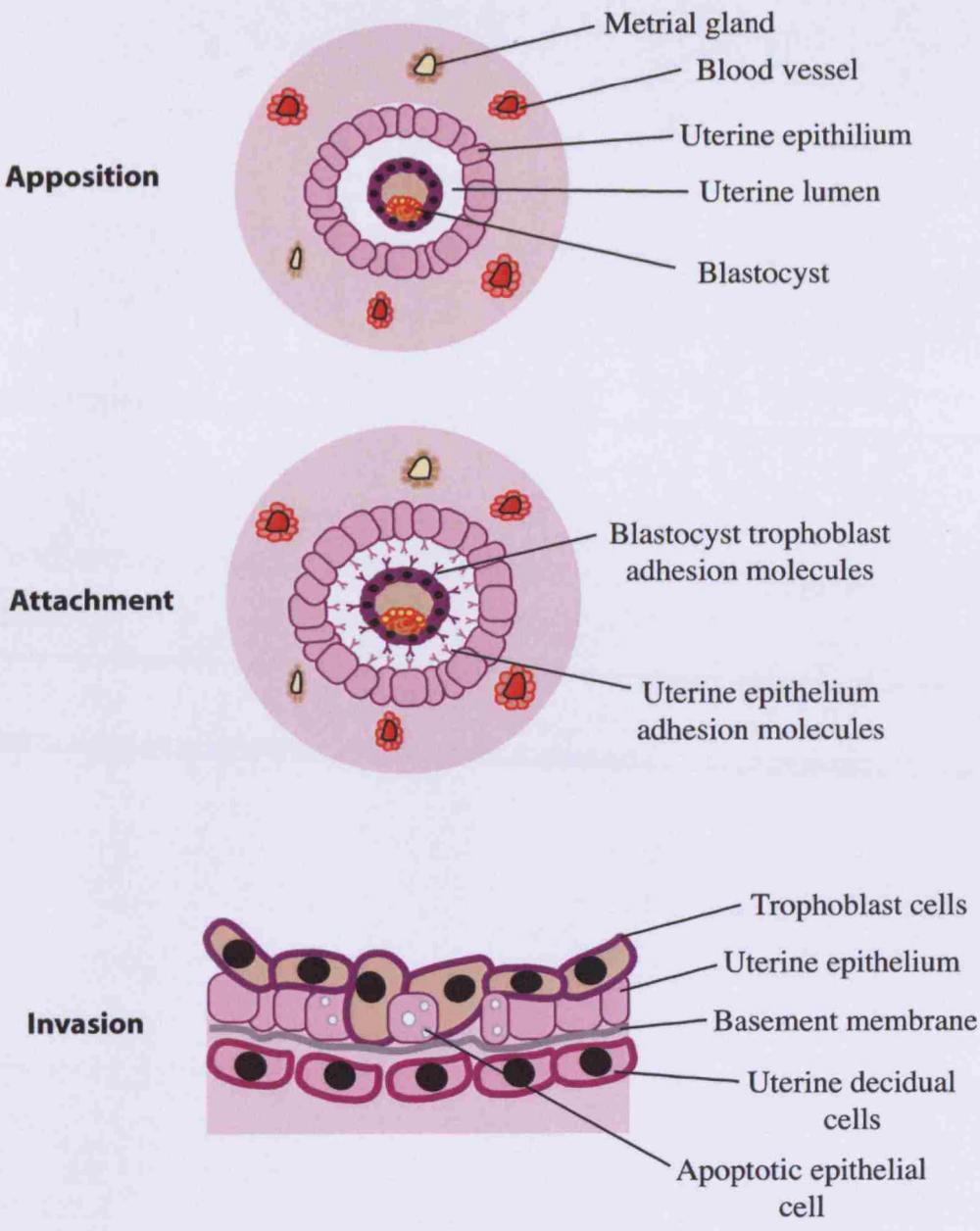


Fig 1.7 Stages of implantation. Implantation of mouse blastocyst showing apposition, attachment and invasion.

Successful attachment depends on the embryo reaching an appropriate stage of development and receptiveness of the endometrium. The mural trophectoderm leads implantation in mice adhering to the uterine wall and attachment involves many molecules including proteoglycans, integrins, selectins, cadherins and other adhesion molecules (Kimber and Spanswick 2000) but also the downregulation of anti-adhesive markers such as MUC-1 (Surveyor, Gendler et al. 1995), which may be key to uterine receptivity. After attachment the uterine epithelial cells proximal to the site of implantation detach from the basement membrane undergo apoptosis allowing trophoblast cells, which phagocytose the cells, to invade the epithelium. Epithelial basement membrane breakdown is facilitated initially by decidual cells but also trophoblast cells, which pause briefly upon invading the epithelium. This process brings developing embryos into contact with the maternal blood supply after about 6 hours of implantation.

Exactly how an embryo implants without triggering an immune response from the mother is an interesting question. This case has been likened to an allograft reaction and is highly unusual and as such must be highly co-ordinated to prevent rejection of the embryo. This process involves establishing a unique environment of chemokines and immune cell types (Fernekorn and Kruse 2005). It is thought that recruitment of regulatory immune cells and exclusion of potentially damaging immune cells could be critical to the success of implantation. This process is regulated by compartmentalisation of the decidua and expression of adhesion molecules such as selectins on endothelial cells. Neutrophils are restricted to necrotic areas of cell digestion at the maternal foetal interface (McMaster, Dey et al. 1993). Macrophages are excluded from the foetal maternal interface (Brandon 1993) and macrophages in the decidua are associated with poor implantation success and macrophage activation markers indicate poor prognosis for pregnancy (Haddad, Duclos et al. 1997).

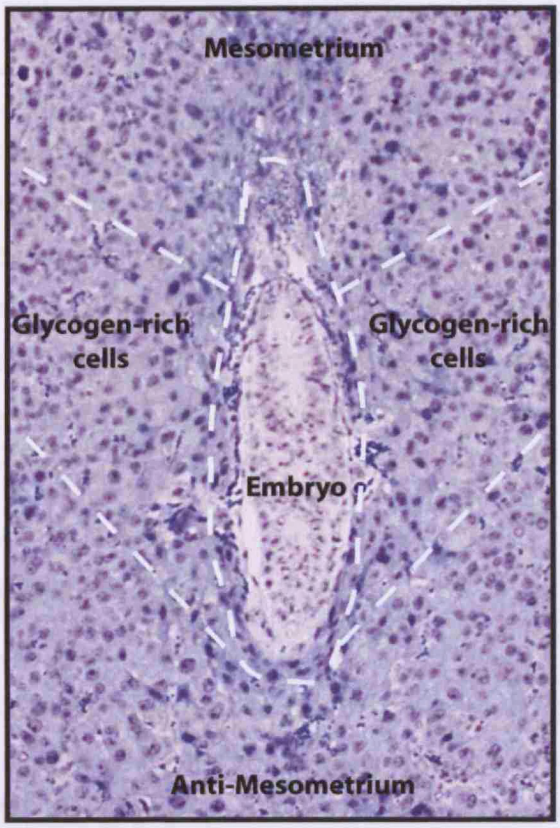
1.6 Postimplantation development

1.6.1 Decidualisation

Implantation of the embryo triggers a massive response in the endometrial stromal fibroblast cells, which proliferate and differentiate in a process called decidualisation (Abrahamsohn and Zorn 1993) as shown in fig 1.8. The process is dependent upon signalling through ovarian steroids in the stroma surrounding the implanting embryo, and results in a new tissue, the decidua, being formed. Decidual cells are larger, more granular and fibrous and have more organelles than undifferentiated stromal cells. Decidualisation begins in the stroma surrounding the implanting embryo, the primary decidual zone, and occurs initially in the sub-epithelial, antimesometrial area (area apposed to the inner cell mass). The first decidual cells are in place when trophoblast cells invade the uterine epithelium. There are regional differences in the extent and timing of decidualisation, which are important to bear in mind for the purposes of this thesis. Decidualisation proceeds through the antimesometrium although the extent of decidualisation recedes as distance from the embryo increases. Decidualisation occurs later (around E 7.5 in mice) in the decidual crypt and mesometrium, which develops into two distinguishable regions a glycogen rich region proximal to the anti-mesometrium and a region of largely undifferentiated stromal cells (Abrahamsohn and Zorn 1993).

This processes of attachment, invasion and spreading of the trophoblast lineages involves many components of the extracellular matrix. Basement membrane and extracellular matrix components such as fibronectin, type IV collagen and laminin can interact with trophoblast cells receptors and promote attachment and growth. Penetration of the basement membrane

A



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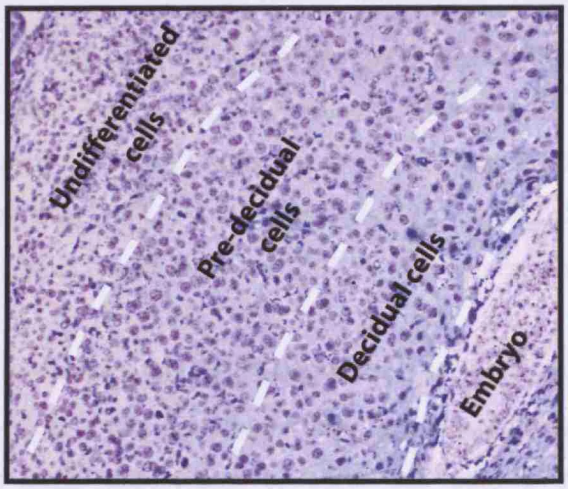


Fig 1.8 Decidualisation in the maternal endometrium. Representation of maternal decidual orientation. (A) Embryo development at E 7.0 with the areas of decidua. (B) Extent of decidualisation in the maternal decidua at E 7.0 showing differentiation gradients.

and invasion of the surrounding stroma is important and must be precisely controlled. Remodelling of the extracellular matrix is largely mediated by matrix metalloproteinases (MMPs) themselves regulated by endogenous tissue inhibitors of MMPs (TIMPs) (Brenner, Adler et al. 1989), which together mediate and restrict the remodelling to ensure the appropriate level of embryo invasion into the uterine stroma and the correct stromal response to invasion.

1.6.2 Placental development

Embryo development postimplantation is highly dependent on the formation and growth of a functioning placenta as shown in fig 1.9. The roles of the placenta include establishing a foetal/maternal interface for exchange of metabolites and gases, waste product removal, hormonal regulation of both maternal and foetal tissues and a successful interaction with the maternal immune system (Cross, Werb et al. 1994; Adamson, Lu et al. 2002). The mature placenta consists of three layers with contributions from both mother and foetus. There is an outer layer of maternal decidual cells and vasculature, an interface layer comprised of foetal trophoblast cells that have invaded the maternal decidua and an inner layer of highly branched foetal villi for efficient exchange.

Placental development begins in the blastocyst with the differentiation of the trophectoderm from the inner cell mass (Sherman 1975) with trophectoderm cells proceeding to form the mature placenta. Upon blastocyst implantation the mural trophectoderm cells, which are not in contact with the inner cell mass, enter a process called endoreplication. The cells stop dividing yet continue replicating their DNA to become polyploid (Gardner and Davies 1993; MacAuley, Cross et al. 1998). These cells increase in size and are called trophoblast giant cells. The polar

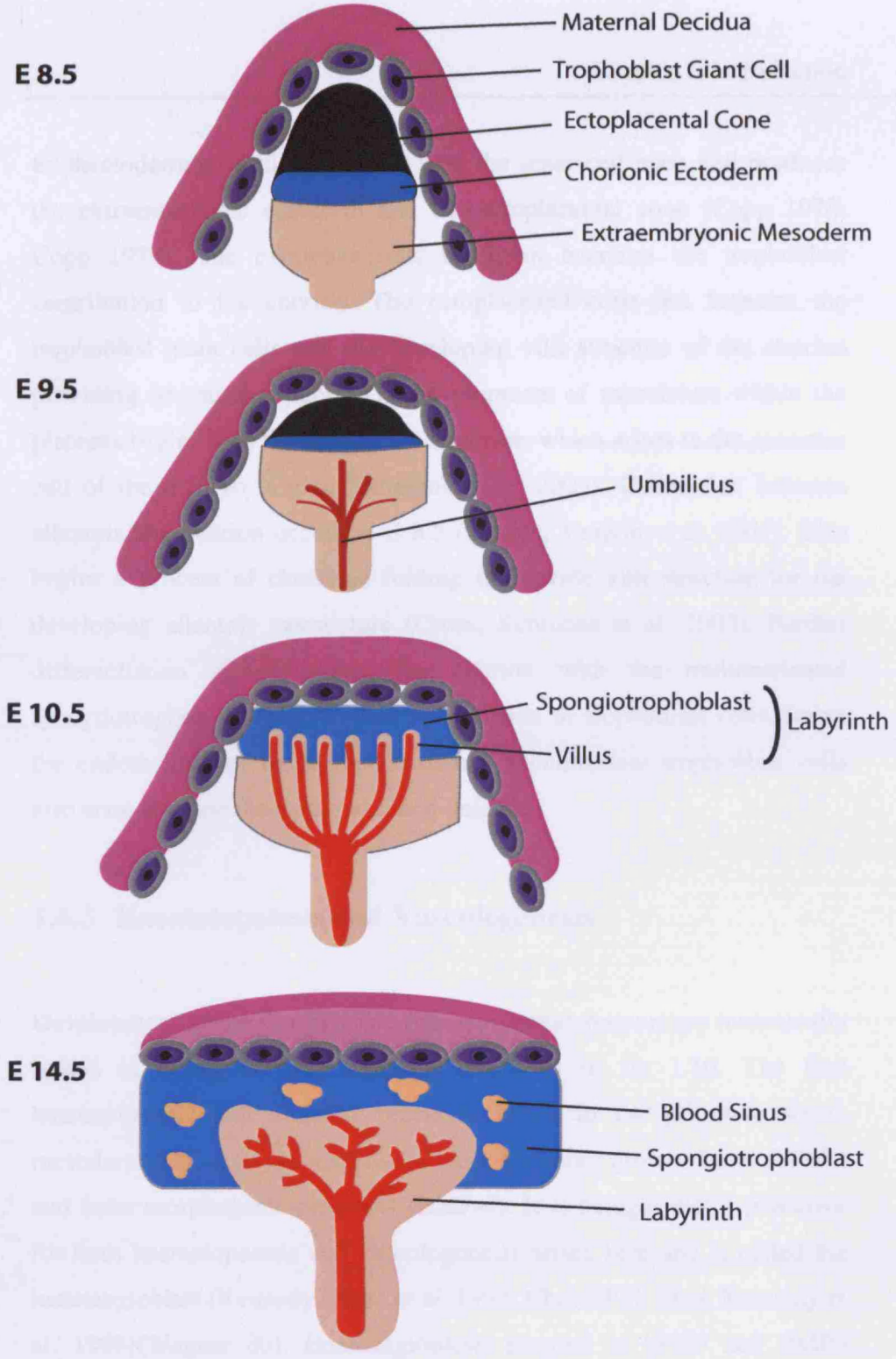


Fig 1.9 Development of the placenta. Development of the placenta in mice through various time stages. showing emergent cell and tissue types.

trophectoderm is in direct contact with the inner cell mass and produces the extraembryonic ectoderm and the ectoplacental cone (Copp 1978; Copp 1979). The extraembryonic ectoderm becomes the trophoblast contribution to the chorion. The ectoplacental cone lies between the trophoblast giant cells and the developing villi structure of the chorion providing structural support. The development of vasculature within the placenta begins with the allantois, a structure, which arises in the posterior end of the embryo (Cross, Simmons et al. 2003). Connection between allantois and chorion occurs at E 8.5 (Downs, Temkin et al. 2001). This begins a process of chorionic folding to provide villi structure for the developing allantois vasculature (Cross, Simmons et al. 2003). Further differentiation occurs within the chorion with the multinucleated syncytiotrophoblast cells, formed from fusion of trophoblast cells, lining the endothelium of foetal blood vessels. Mononuclear trophoblast cells also arise that line the maternal blood vessels.

1.6.3 Haematopoiesis and Vasculogenesis

Development of the vascular and haematopoietic systems are intrinsically linked in embryonic development as shown in fig 1.10. The first haematopoietic and vascular precursors arise in the primitive streak mesoderm (Huber 04) in response to basic fibroblast growth factor (bFGF) and bone morphogenic protein 4 (BMP-4). It is thought that a precursor for both haematopoiesis and vasculogenesis arises here and is called the haemangioblast (Kennedy, Firpo et al. 1997; Choi 1998; Choi, Kennedy et al. 1998)(Wagner 80). Haemangioblasts respond to bFGF and BMP4 signalling becoming positive for vascular endothelial growth receptor-2 (VEGFR-2) also called Flk-1 (Millauer, Wizigmann-Voos et al. 1993; Choi 1998). While there is substantial evidence for the existence of a dual haematopoietic and vascular precursor recent studies have raised the

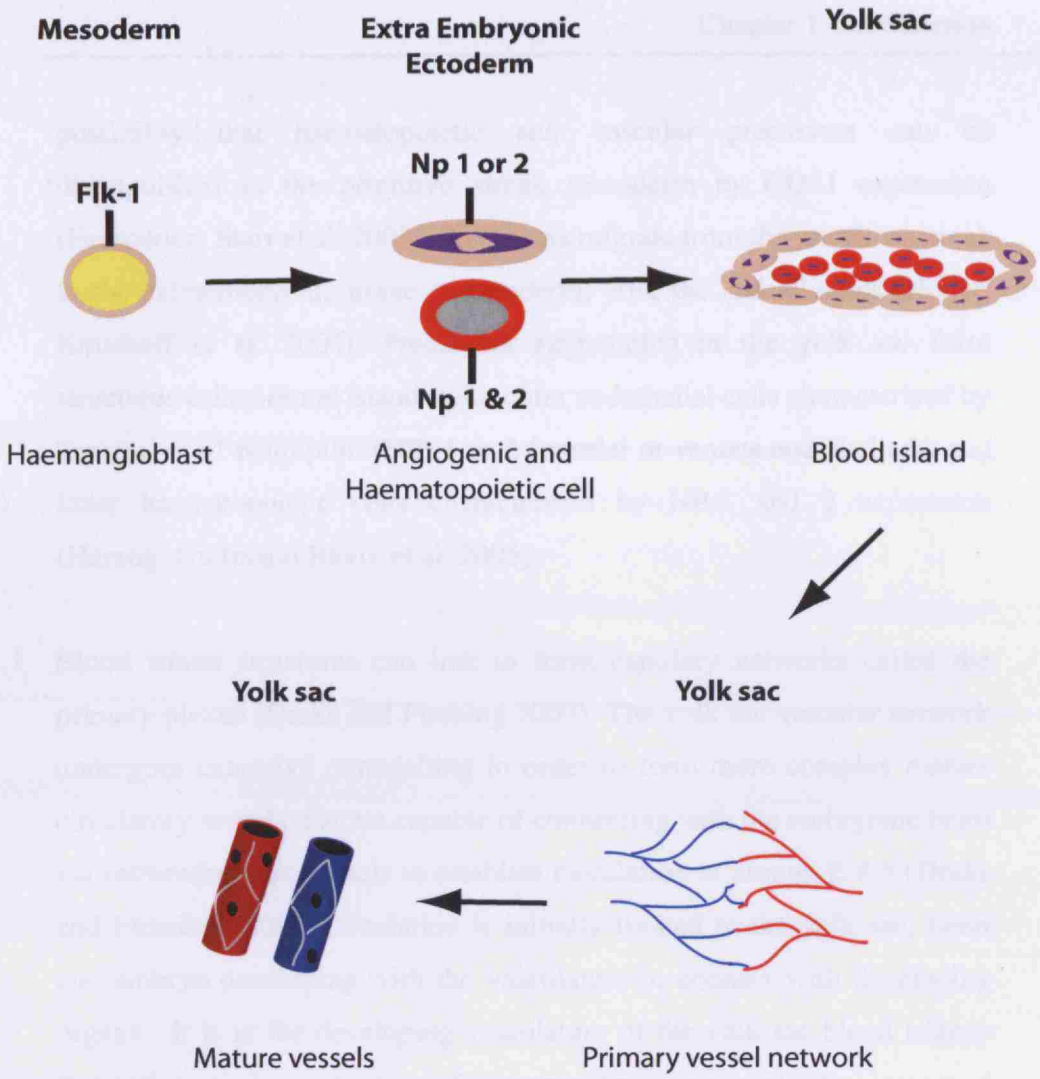


Fig 1.10 Vasculogenesis and Haematopoiesis in development. Development of haematopoietic precursors from flk-1 positive mesodermal cells (Yellow) and progress to NP-1 (arterial) and NP-2 (Venous) angiogenic precursors (Purple) and haematopoietic precursors (Red). FLk-1 - vascular endothelial growth factor receptor, NP - neuropilin.

possibility that haematopoietic and vascular precursors can be distinguished in the primitive streak mesoderm by CD41 expression (Ferkowicz, Starr et al. 2003). Precursors migrate from the primitive streak to the extraembryonic tissue of ectoderm, yolk sac and allantois (Huber, Kouskoff et al. 2004). Precursors aggregating in the yolk sac form structures called blood islands with outer endothelial cells characterised by expression of neuropilin (NP) 1 or 2 (arterial or venous endothelium) and inner haematopoietic cells characterised by NP-1 and 2 expression (Herzog, Guttmann-Raviv et al. 2005).

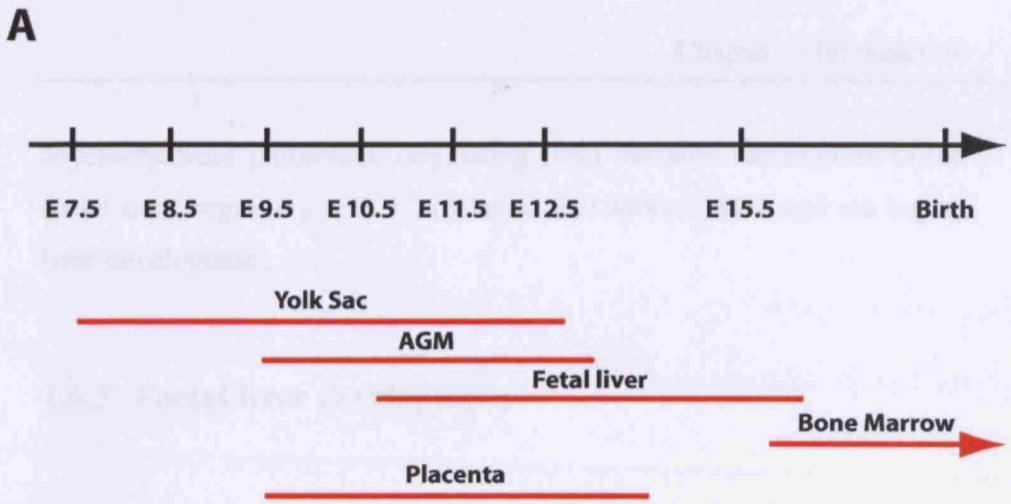
Blood island structures can link to form capillary networks called the primary plexus (Drake and Fleming 2000). The yolk sac vascular network undergoes extensive remodelling in order to form more complex mature circulatory vessels that are capable of connecting with the embryonic heart via intraembryonic vessels to establish circulation at around E 8.5 (Drake and Fleming 2000). Circulation is initially limited to the yolk sac, heart and embryo developing with the vasculature to connect with developing organs. It is in the developing vasculature of the yolk sac blood islands that embryonic haematopoiesis first occurs from precursor cells.

1.6.4 Haematopoietic development

In adults haematopoiesis involves constant production of haematopoietic cells from haematopoietic stem cells (HSCs) in the bone marrow. Haematopoietic stem cells are defined by ability to produce multiple blood cell types and the ability to self renew. The way to test for HSCs is their ability to repopulate a lethally irradiated mouse. In embryogenesis HSCs develop in a complex way with multiple sites of origin coupled with a progressive change in HSC location until colonisation of the bone marrow prior to birth brings us to the adult state (Cumano and Godin 2007) as

shown in fig 1.11. The first blood cells arise from mesodermal precursors, which migrate to form the yolk sac blood islands making the blood islands the first embryonic site of haematopoiesis (Moore and Metcalf 1970). These primitive precursors produce only primitive erythrocyte cells, which are characterised by their nucleus, large size and expression of both foetal and adult globin genes (Haar and Ackerman 1971; Ingram 1972; Ferkowicz, Starr et al. 2003). Primitive erythrocytes are generated between E 7.0 and E 8.5 and are gradually replaced by myeloerythroid precursors in the yolk sac (Cumano, Dieterlen-Lievre et al. 1996). Myeloerythroid precursors produce definitive erythrocytes, which can be characterised by their smaller size, expression of adult globin genes and loss of nucleus in the circulation.

None of the early yolk sac progenitors are truly HSCs as they cannot produce all the blood cell types and are relatively short lived with no repopulation ability. It is not clear whether the yolk sac gives rise to HSCs or is a potential host site to HSCs when they arise (Cumano, Dieterlen-Lievre et al. 1996; Cumano, Ferraz et al. 2001). More recently studies have shown that the placenta is also a site of haematopoiesis as early as E 9.0 and could also generate HSCs (Alvarez-Silva, Belo-Diabangouaya et al. 2003). It is very clear that HSCs arise within the embryo proper in a region called the para aortic splanchnopleurae (pSP), which later develops into the aorta-gonad-mesonephros (Medvinsky, Samoylina et al. 1993; Medvinsky and Dzierzak 1996). HSCs can be found in this region at E 10.5 and have been shown to originate from that site. The AGM HSCs are thought to be a small population of HSCs but that they contribute predominantly to adult bone marrow HSCs (Lassila, Eskola et al. 1978). One of the reasons for controversy over the origins of HSCs is that it is known that AGM HSCs can migrate to the haematopoietic sites of the yolk sac and placenta. Development of haematopoietic cells continues with migration from early sites to later and more mature tissues.



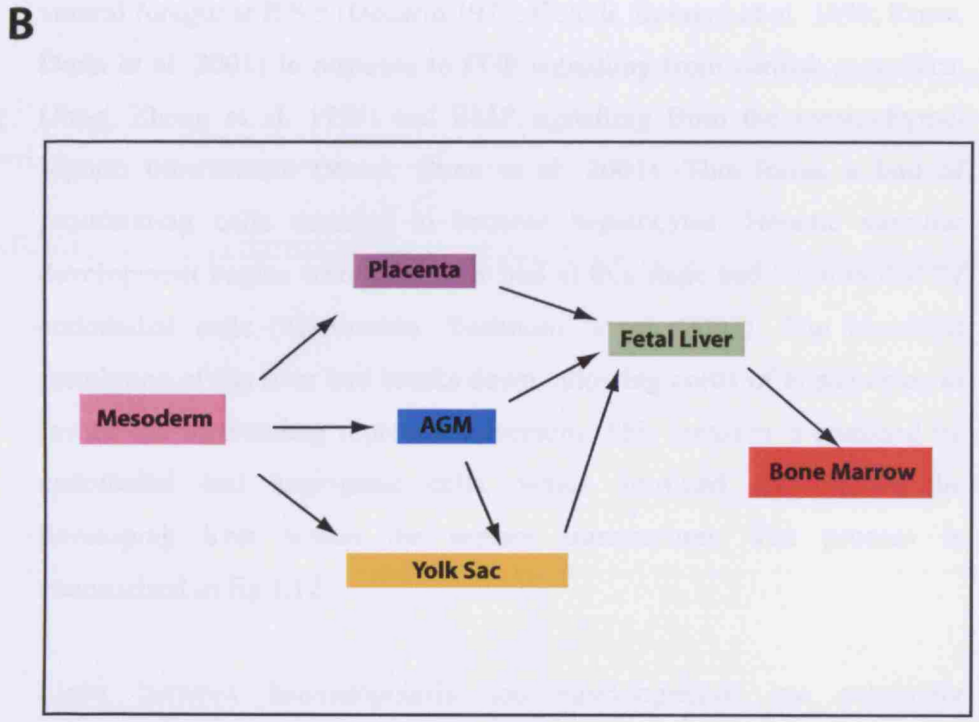


Fig 1.11 Timing and location of embryonic haematopoiesis. Timing of haematopoietic onset in embryonic tissues (A) at embryonic stages (E) days post coitum. Migration of haematopoietically fated cells in developing embryo (B) includes known migrations and suspected migrations like AGM to foetal liver. Key, AGM = Aorta-Gonad-Mesonephros.

Myeloerythroid precursors, originating from the yolk sac migrate to the foetal liver beginning at E 9.5 (Johnson and Moore 1975) and are key to liver development.

1.6.5 Foetal liver development

In mice, the liver develops as an endodermal invagination formed from the ventral foregut at E 8.5 (Douarin 1975; Gualdi, Bossard et al. 1996; Rossi, Dunn et al. 2001) in response to FGF signalling from cardiac mesoderm (Jung, Zheng et al. 1999) and BMP signalling from the mesenchymal septum transversum (Rossi, Dunn et al. 2001). This forms a bud of proliferating cells destined to become hepatocytes. Hepatic vascular development begins within the liver bud at this stage and is promoted by endothelial cells (Matsumoto, Yoshitomi et al. 2001). The basement membrane of the liver bud breaks down, allowing cords of hepatocytes to invade the surrounding septum transversum. This invasion is confined by endothelial and angiogenic cells, which surround and define the developing liver within the septum transversum. The process is summarised in fig 1.12.

Links between haematopoiesis and vasculogenesis are extremely important as the developing and connecting vascular system allows migration of haematopoietic cells. The first circulation is established between the heart, dorsal aorta and yolk sac at E 8.5. The heart starts to beat and predominantly erythrocytes enter the circulation. Migration of haematopoietic cells has been shown to be of vital importance in the developing liver providing key maturation signals (Kamiya, Kinoshita et al. 1999; Kinoshita, Sekiguchi et al. 1999). At E 9.5 myeloerythroid haematopoietic precursor cells migrate to the foetal liver, primarily from the yolk sac, where they proliferate and differentiate. HSCs first arrive in

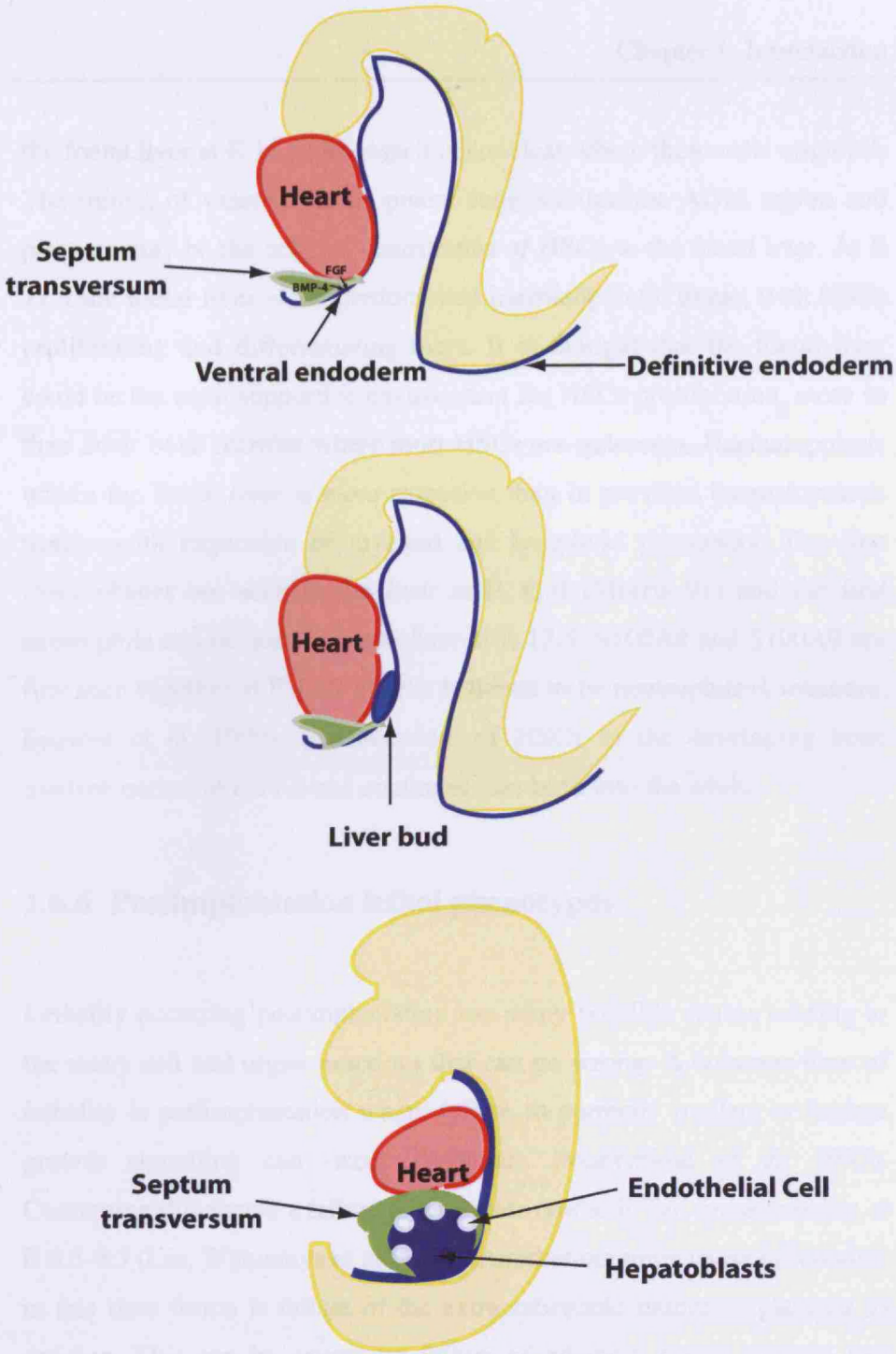


Fig 1.12 Liver organogenesis in the developing embryo. Development of the embryonic liver from ventral endoderm (blue) in response to signalling from the septum transversum (Green) and the cardiac mesoderm (red). Development of liver bud (blue and invasion of the septum transversum with the assistance of endothelial cells (white). Other embryonic tissues are shown (yellow) to give context to liver development.

the foetal liver at E 11.5 although it is not clear where these cells originate. The timing of vascular development suggests that the AGM region and placenta may be the original contributors of HSCs to the foetal liver. At E 12.5 the foetal liver is the predominant haematopoietic tissue, with HSCs proliferating and differentiating there. It is thought that the foetal liver could be the most supportive environment for HSCs proliferation, more so than adult bone marrow where most HSCs are quiescent. Haematopoiesis within the foetal liver is more extensive than in previous haematopoietic tissues with expansion of myeloid and lymphoid precursors. The first macrophages are seen in the liver at E 11.0 (Morris 91) and the first neutrophils can be seen in foetal liver at E 12.5. S100A8 and S100A9 are first seen together at E 12.0 in cells believed to be neutrophils (Lichanska, Browne et al. 1999). Establishment of HSCs in the developing bone marrow occurs at E 17.5 and continues past birth into the adult.

1.6.6 Postimplantation lethal phenotypes

Lethality occurring postimplantation has many possible causes relating to the many cell and organ functions that can go wrong. A common time of lethality is periimplantation where failure to correctly implant or initiate growth signalling can occur (Feldman, Poueymirou et al. 1995). Continuing this theme a failure to gastrulate correctly can cause lethality at E 6.5-9.5 (Liu, Wakamiya et al. 1999). Another common cause of lethality in this time frame is failure of the extraembryonic tissues or placenta to develop. This can be caused by failure of allantoic fusion (Naiche and Papaioannou 2003), failure of vasculogenesis (Shalaby, Rossant et al. 1995) or failure of extraembryonic membrane function (Morasso, Grinberg et al. 1999). Failure of cardiac development is common between E 7.5 and E 10.5 (Lin, Schwarz et al. 1997). Lethality due to yolk sac defects can also be seen (Davenport, Jerome-Majewska et al. 2003). With

so many possible causes of lethality it is important to analyse a model closely. It has also been known for a null lethal phenotype to be affected by mouse genetic background.

1.7 Aims of this thesis

The purpose of this thesis is to re-examine the role of S100A8 in development and to determine whether it needs to be reassessed following on from the work of Passey et al 1999. One aim is to discover what the non-redundant role of S100A8 might be. Is S100A8 expressed independently of S100A9, and if so how is it stabilised. It is also considered that revealing the role of S100A8 in development might provide insights into the role of the S100A8 and S100A9 heterodimer in the adult animal.

CHAPTER 2

2 Materials and Methods

2.1 Materials

2.1.1 Buffers/Serum

Reagent	Further Information
FACS wash	PBS containing 0.2% bovine serum albumin
FACS fix	PBS containing 2% formaldehyde
Saponin Buffer	PBS containing 0.5 bovine serum albumin and 0.5% saponin
HBSS	Hanks Balanced Salt Solution (Gibco)
DMEM	Dulbecco's modified Eagle's medium (Sigma)
HEPES	(Gibco)
M16	Embryo culture buffer (Sigma)
M2	Embryo culture buffer (Sigma)
IMDM	Iscove's modified Dulbecco's medium (Gibco)
FCS	Foetal calf serum (PAA cell culture)

2.1.2 Antibodies and detection reagents

Antibody	Conjugate	Epitope	Species	Supplier
NH9		S100A8	Rabbit	CRUK

Calgranulin A		C-terminal peptide of S100A8	Goat	Santa Cruz
6A4		S100A8	Rat	CRUK
2B10		S100A9	Rat	CRUK
F4/80		160kD macrophage glycoprotein	Rat	Serotec
7/4		Neutrophil/ monocyte 40kD protein	Rat	Caltag Medsystems
IgG2a		Y13	Rat	CRUK
IgG2b		PyLT-1	Rat	CRUK
Rabbit anti rat	HRP	IgG	Rabbit	Southern Biotechnology
Goat anti Rabbit	HRP	IgG	Goat	Dako-Cytomation
Rabbit anti mouse	HRP	IgG	Rabbit	Dako-Cytomation
Goat anti rabbit	Alexa 488	IgG	Goat	Sigma
Goat anti rabbit	Alexa 568	IgG	Goat	Sigma
Rabbit anti goat	Alexa 488	IgG	Rabbit	Sigma
Goat anti rat	FITC	IgG	Goat	Jackson Immunoresearch
Rabbit anti goat	FITC	IgG	Rabbit	Sigma
Goat anti rabbit	FITC	IgG	Goat	Sigma

2.1.3 Gifts

Embryonic stem (ES) cell derived cultures differentiated down erythrocyte lineage – Valerie Kouskoff (Kouskoff, Lacaud et al. 2005). 10^6 cell samples for RT-PCR and 10^7 cells for western blot analysis.

2.2 Methods

2.2.1 Animal husbandry

S100A9 (Hobbs 03) and S100A8 null mice were generated by R.May in the Leukocyte Adhesion Laboratory. Transgenic SV129 x C57BL/6J mice were derived from two independent clones for each strain and backcrossed against C57Bl/6J mice to a minimum of 6 generations. Offspring were genotyped using the PCR methods outlined in 2.2.2. Mice were maintained in a pathogen-free environment and all procedures were in accordance with Home Office guidelines.

2.2.2 Genotyping

2.2.2.1 Tailsnip preparation

Mouse tailsnips of approx 5mm were cut into Eppendorf tubes and digested in 700 µl of tail digestion buffer (50mM Tris pH 8, 100mM EDTA, 100mM NaCl, 1% SDS, 25µl 10mg/ml proteinase K) at 55°C overnight. Samples were boiled at 95°C for 15 mins and then spun at full speed in a microfuge for 10 mins at 4°C. 400µl supernatant was added to an Eppendorf tube with 400µl isopropanol. 80µl 3M sodium acetate is added and the Eppendorf inverted several times vigorously to mix the solutions. Eppendorf spun at 4°C for 20 mins. Supernatant was aspirated and the pellet washed with 500µl 70% ethanol. The Eppendorf tubes were spun for 20 mins and the supernatant aspirated. Eppendorf tubes were left to air dry for approx 2 hrs at RT prior to resuspension in 20 µl water.

2.2.2.2 Preimplantation embryo DNA preparation

Preimplantation embryos were generated by the CRUK Transgenic Services

unit as detailed in 2.2.10. Embryos were snap frozen in 1µl DDW in individual Eppendorf tubes. Embryos were defrosted by adding 5µl 4°C lysis buffer (5mM Tris pH8, 0.1% SDS, 2mM NaCl + 1U Proteinase K; Promega) to frozen Eppendorf tubes. The Eppendorf tubes were spun down and the samples transferred to PCR wells to undergo digestion in a PCR cycle machine with the following programme.

Time	Temperature °C	Cycles
60 mins	60	1
5 mins	95	1
Holding	4	forever

1µl of digested sample was used as DNA template in 2-step PCR reactions.

2.2.2.3 Postimplantation embryo DNA preparation

Postimplantation embryos were generated from timed matings. Mice were euthanised by asphyxiation followed by cervical dislocation. Embryos were extracted from maternal decidua using a dissecting microscope and then digested using the same protocol for tailsnip DNA preparation in 2.2.2. DNA is resuspended in 20 µl water with 1µl used as template for the 1-step PCR protocol outlined in 2.2.2.

2.2.2.4 1-step PCR process

1µl of DNA template is mixed with 1.5 U TAQ polymerase (CRUK), 125ng of each primer (S100A8 2nd reaction primers for S100A8 genotyping, S100A9 primers for S100A9 genotyping) and PCR buffer (1 x Thermophilic PCR buffer, 1.5 mM $MgCl_2$, 200µM dATP/dGTP/dTTP/dCTP; Promega) to a final volume of 25 µl. Samples are amplified using Peltier Thermal Cycler (MJ Research). The basic cycle programme is shown below.

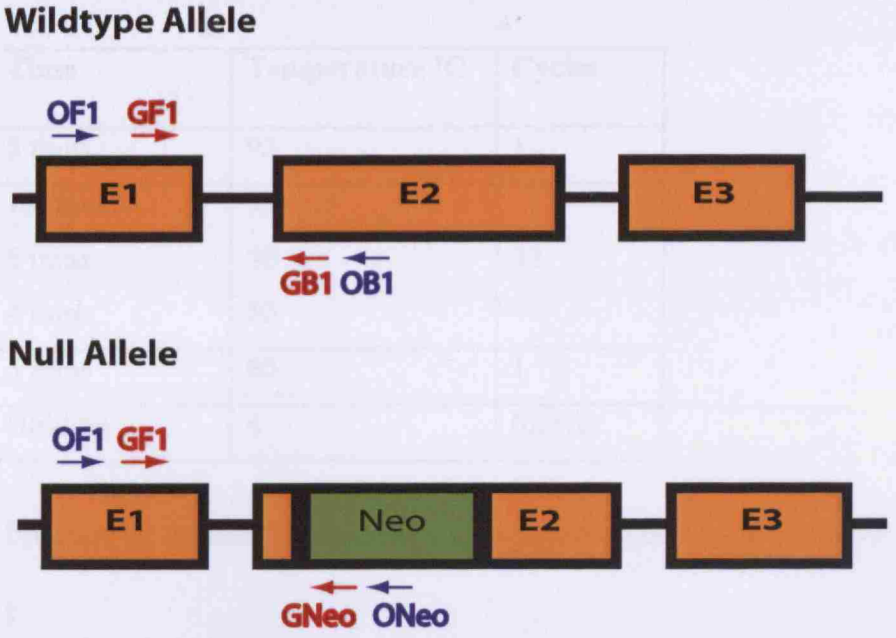
Time	Temperature °C	Cycles
5 mins	95	1
30s 30s 60s	95 55 72	32 (typical)
5 mins	72	1
Holding	4	forever

2.2.2.5 2 Step PCR protocol

1µl of each DNA sample was mixed with 1.5 U TAQ polymerase (CRUK), 125ng of each primer and PCR buffer (1 x Thermophilic PCR buffer, 1.5 mM $MgCl_2$, 200µM dATP/dGTP/dTTP/dCTP; Promega) to a final volume of 25 µl. The first round of PCR used S100A8 1st reaction PCR primers (as shown in fig 2.1) using the programme shown below.

Time	Temperature °C	Cycles
5 mins	95	1
10 mins 5 mins 4 mins	95 55 60	24
5 mins	60	1
Holding	4	forever

Products undergo a second PCR cycle using 1µl of 1st reaction round product as DNA template and using S100A8 2nd reaction primers (as shown in fig 2.1) in an otherwise identical reaction mixture. The samples were placed in the programme shown below.



1st reaction primers	OF1 OB1 ONeo
2nd reaction primers	GF1 GB1 GNeo

Fig 2.1 1st and 2nd PCR reaction primers in relation to S100A8 gene structure. Primer combination for 1st reaction (blue) and 2nd reaction (red) shown in relation to wildtype and null allelles.

Time	Temperature °C	Cycles
5 mins	95	1
10 mins 5 mins 4 mins	95 50 60	32
5 mins	60	1
Holding	4	forever

Products of the final reaction are run on agarose gels as shown below in 2.2.2.

2.2.2.6 PCR Primers

All primers were synthesised by Sigma-Genysis

S100A8- 2nd reaction Primers
GF1- AGCCTCACATATCCTTTGTCA
GB1-GACATCAATGAGGTTGCT CAA
GNEO-ACCGCTTCCTCGTGCTTTACG

S100A8- 1st reaction primers
OF1- GCAGCTGACACTTAGCCTCAC
OB1- CCATCCCAGCACCATTAGAA
ONEO-ACATAGCGTTGGCTACCCGTG

S100A9 primers
GF2-AACATCTGTGACTCTTTAGCC
GB2-CATCTGAGAAGGTGCTTTGTT
GNEO-ACCGCTTCCTCGTGCTTTACG

2.2.2.7 Agarose gel electrophoresis

TAE buffer (from a 50 x solution containing 242g Tris-base, 100ml 0.5M EDTA, 57.1ml glacial acetic acid made up to 1 l, pH8) containing 1.8% agarose (Invitrogen) was heated by microwave to dissolve the agarose (approx 3 mins). Ethidium bromide was added (5µl/100ml; Sigma) before pouring the mixture into a gel mould with lane marker combs. Blue/Orange loading buffer (Promega) was added at a ratio of 1:10 to PCR products before loading to track progress. PCR products were electrophoresed at 100-120mV in 1 x TAE buffer with 5µl/L ethidium bromide. Bands were visualised by UV illumination.

2.2.3 DNA sequencing

PCR bands were cut from agarose gels and DNA purified using Qia quick gel extraction kit (Qiagen) according to manufacturers instructions. 10ng of DNA template was added to PCR tubes along with 150ng of template primer, 8µl terminator ready reaction mix (Qiagen) and water to a final volume of 20µl. PCR cycling was performed as follows.

Time	Temperature °C	Cycles
5 mins	95	1
10 mins 5 mins 4 mins	95 55 60	25
5 mins	60	1
Holding	4	forever

Reaction products were prepared for sequencing by purification using a DyeEx spin column (Qiagen). Purified reaction products are dried and resuspended in 20µl Hi Di formamide. 15µl of sample were loaded into 96 well plates and denatured at 96 °C for 2 mins. The samples were then loaded

into a capillary sequencing Prism 3730 machine (Applied Biosytems).

2.2.4 SDS-PAGE

Samples for SDS-PAGE were prepared by addition of lysis buffer (7 M urea, 10% glycerol, 10 mM Tris–HCl (pH 6.8), 1% sodium dodecyl sulphate (SDS), 5 mM dithiothreitol (DTT), 0.5 mM phenylmethyl sulfonyl fluoride (PMSF) with 1 mg/l aprotinin, pepstatin and leupeptin) and pipetted using a 20-gauge 0.9mm needle with a 2ml syringe. The sample is then spun down and the supernatant stored at -20°C. SDS-PAGE was conducted using the NuPAGE precast gel system (Invitrogen) and specifically 10% Bis-Tris Gels run with MOPS buffer (Invitrogen). Lysates were diluted 1:1 with loading buffer (2% SDS, 0.5M Tris pH6.8, 25% glycerol, bromophenol blue and 1% mercaptoethanol). Samples were loaded in each well and run for 1hr at 180V.

Following SDS-PAGE, proteins were transferred to a nitrocellulose membrane (Hybond ECL; Amersham Biosciences) at 60 V for 1 hr in a Transblot cell (Biorad) in transfer buffer (25mM Tris, 192mM Glycine, 20% Methanol). Ponceau S solution (Sigma) is used to confirm transfer before blocking in PBS/Tween (PBS + 0.1% Tween 20) containing 5% milk powder either for 1 hr at RT or overnight at 4°C. Membranes are then incubated at RT for 1hr with 1° antibody in PBS/Tween + 5% milk. After 3 washes in PBS/Tween membranes were then incubated with a HRP conjugated 2° antibody in PBS/Tween + 5% milk. After a further 3 washes with PBS/Tween, bound antibody is visualised using ECL chemiluminescent detection kits (Amersham Biosciences) for 1 min at RT, followed by exposure to film (Hyperfilm; Amersham Biosciences). S100A8 was detected using antibodies (Ab) NH9 (1:2000, CRUK antibody service) and Calgranulin A (1:2000; Santa Cruz) followed by anti-rabbit HRP (1:7000; Dako Cytomation) or anti-goat HRP (1:7000; Dako cytomation). Each polyclonal antibody was compared to control species serum. S100A9 was detected using monoclonal antibody (mAb) 2B10 (1:2000; CRUK antibody service) followed by anti-rat HRP (1:7000; Southern Biotechnology). 2B10 was compared with control IgG2a

mAb.

2.2.5 2.2.5 Immunohistochemistry

For immunohistochemistry, samples were fixed in neutral buffered formalin (NBF; 10% formalin, 45mM Na_2HPO_4, 29mM $NaH_2PO_4.H_2O$, pH7) for 24 hr before being transferred to 70% ethanol solution. Samples were then embedded in paraffin before cutting. Sections of 8 μm were cut from fixed, embedded tissue blocks and placed on glass slides. Sections are dewaxed using xylene and hydrated using a succession of 100% ethanol, 70% ethanol and DDW steps. Endogenous peroxidase is blocked before sections undergo antigen retrieval methods (10 mins in 10°C citrate buffer, 10 mins in 1 mg/ml trypsin or no treatment). Sections are cooled for 20 mins and then placed in PBS buffer. Sections are then blocked using normal serum. Primary antibody was applied for 1hr followed by 3 washes (all washes in PBS). Secondary antibody is applied for 45 mins followed by 3 washes. Sections are then treated with avidin-biotin complex (ABC) reagent (Vector) for 30 mins followed by 3 washes. Sections are stained with 3,3'-diaminobenzidine (DAB) stain for approx 1-2 mins and then soaked in DDW to prevent further DAB action. Slides were counterstained in Harris' haematoxylin solution for 3 mins followed by dehydration using a sequence of DDW, 70% EtOH and 100% EtOH steps. Sections are rinsed in xylene and coverslips applied using mounting solution. S100A8 was detected using mAb 6A4 (1:50; CRUK), S100A9 using mAb 2B10 (1:50; CRUK), macrophages using mAb F4/80 (1:50 Serotec) and neutrophils and monocytes using 7/4 (1:50; Caltag Medsystems). Secondary Ab used was anti-rat biotinylated (1:400; Vector) for mAbs 6A4, 2B10, F4/80 and 7/4. mAbs were compared with appropriate control IgG isotype mAb.

2.2.6 2.2.6 Flow cytometry

$1x10^6$ cells in 20μl were placed in each well. Cells were fixed for 15 mins

using FACS fix (2% PFA in PBS). Cells were then washed 3 times in FACS wash (PBS containing 0.2% BSA) before resuspension in 0.1M Glycine solution for 10 mins. A further 3 washes were performed before cells were permeabilised and blocked by incubation in 100µl saponin buffer (PBS containing 0.5% saponin and 0.5% BSA) with 1% normal serum for 30mins. 100µl of saponin buffer with 1° antibody was added and incubated for an additional 45 mins (mAbs 6A4 and 2B10 (CRUK), Abs calgranulin A (Santa-Cruz), and NH9 (CRUK) were all used at 1:100). Cells were washed 3 times in saponin buffer before incubation with 2° antibody in saponin buffer for 1hr (secondary Ab for mAbs 6A4 was goat-anti rat FITC conjugated (Jackson Immmunoresearch), for calgranulin A, rabbit anti-goat FITC conjugated (Sigma), and for NH9 goat anti rabbit FITC conjugated (Sigma) were all used at 1:400). Finally cells were washed 3 times in saponin buffer before resuspension in FACS fix solution ready for analysis. Cells were analysed using a FACScalibur machine (BD biosciences)

2.2.7 2.2.7 *In situ* hybridisation

In situ hybridisation in this thesis was conducted by CRUK *in situ* hybridisation service. For *in situ* hybridisation samples were fixed in neutral buffered formalin (NBF; 10% formalin, 45mM Na_2HPO_4, 29mM $NaH_2PO_4.H_2O$, pH7) for 24 hr before being transferred to 70% ethanol solution. Samples were then embedded in paraffin. Localisation of S100A8 and S100A9 mRNA was shown by *in situ* hybridisation. Complementary antisense mRNA probes for the full-length cDNA sequences of S100A8 and S100A9 were labelled with 35S-UTP (800Ci/mM; Amersham). All *in situ* hybridisation was conducted on 4µm serial section of formalin fixed and paraffin embedded decidual tissues. The *in situ* method involves pre-treatment, hybridisation, washing and dipping of slides in photographic emulsion for autoradiography and has been previously described (Senior 1988). Autoradiography was carried out at 4°C for 3-10 days before developing and counterstained with Giemsa.

2.2.8 2.2.8 RT-PCR

RNA was isolated using the RNeasy kit (Qiagen) according to the manufacturer's instructions. Once purified, RNA was eluted into 40µl of RNAse free water. RNA was used to create cDNA using the First Strand cDNA Synthesis Kit (GE healthcare) according to manufacturers instructions. A random hexamer primer was provided in the kit and was used for all cDNA production. The cDNA produced was then used as a DNA template for PCR reactions as described in section 2.2.2.

2.2.9 2.2.9 Tissue sample generation

2.2.9.1 Murine bone marrow extraction

Murine femur and tibia bones were harvested and separated from soft tissues using a scalpel. Bone marrow was flushed into a Petri dish in HBSS buffer containing 0.2% BSA using a needle and 1ml syringe. Bone marrow cells were suspended using repeated pipetting and passed through a cell filter into a 15ml Eppendorf. Cells were pelleted at 15,000rpm for 5mins. The supernatant was discarded and the cells resuspended in Erylyse (0.144M NH4CL/0.017M Tris HCl, pH 7.0) for 3 mins to destroy erythrocytes. The cells were pelleted again and the supernatant discarded. Cells were resuspended in HBSS buffer ready for use in western blot as illustrated in 2.2.4 or in flow cytometry as shown in 2.2.6 or in RT-PCR as shown in 2.2.8.

2.2.9.2 Decidua generation

Decidual tissue was generated from timed matings where vaginal plugs indicate a conception of midnight the evening before detection. The morning the plug is discovered is assigned E 0.5. Pregnant female mice of the correct embryonic stage were euthanised by carbon dioxide asphyxiation followed by cervical dislocation. The uterine horns were dissected and placed in PBS.

Decidual tissue samples were extracted under a dissecting microscope and placed in PBS ready for the next stage. Decidual tissue was lysed for western blot as shown in 2.2.4 or prepared for RT-PCR as outlined in 2.2.8.

2.2.9.3 Yolk sac generation

Yolk sac samples were generated from timed matings. Mice were euthanised at the correct stage and uterine horns were dissected and individual embryo-decidua units isolated in PBS. Yolk sac tissue was isolated from decidua and embryo by physical manipulation under a dissecting microscope. Isolated yolk sac tissues were held in PBS before either lysis for western blot analysis as shown in 2.2.4, passed through a cell filter into HBSS for flow cytometry as shown in 2.2.6 or prepared for RT-PCR as shown in 2.2.8.

2.2.10 Embryo and oocyte generation

Embryos were generated from timed matings by the Transgenic Services at CRUK. Plugs confirmed embryo status at embryonic stage E 0.5. Mice were euthanised at appropriate embryonic stage and dissected using sterile technique. The uterus was trimmed free of fatty tissue and placed in a Petri dish. The uterine horns were isolated and flushed from both ends using DMEM with HEPES medium. Embryos in the Petri dish were then pooled into small drops of M16 media ready for the next stage including genotyping as detailed in 2.2.2, *in vitro* culture shown below in 2.2.10 and fixation for confocal microscopy as shown in 2.2.11. Oocytes were collected from the ovaries of mice primed with equine chorionic gonadotrophin (eCG) and held in M2 medium prior to fixation as shown in 2.2.11.

2.2.10.1 Embryo culture

Embryos were cultured in Petri dishes with M16 medium, covered with mineral oil and placed in an incubator at 37°C with 5% CO_2. Embryos were monitored at 10am and 5pm each day of culture and records kept.

2.2.11 Embryo and oocyte staining for confocal staining

Embryo and confocal staining was conducted in collaboration with the laboratory of John Carroll (UCL). Isolated embryos and oocytes were transferred from M16 medium and fixed in 3.7% paraformaldehyde in PBS solution for 30 mins at RT before being washed in PBS and held in M16 buffer before staining. Fixed embryos and oocytes were washed in PBS before permeabilsation in PBS + 0.25% Triton X100 (Sigma) for 30 mins. After washing in PBS, embryos and oocytes were blocked using PBS + 2% BSA along with an appropriate normal serum for 2 hrs. Embryos were then washed before incubation with PBS and 1° antibody 1:100 at 4°C overnight (Calgranulin A, NH9 or GM-130; each at 1:100). Samples were then washed 3 times in PBS before incubation with 2° antibody for 1hr at 37°C (Alexa Fluor 488 rabbit anti-goat (Sigma) for calgranulin A, Alexa Fluor 568 goat anti-rabbit (Sigma) for GM-130 and Alexa Fluor 488 goat anti-rabbit (Sigma) for NH9 each at 1:400). After washing in PBS, all samples were counterstained with 5μg/ml Hoechst 33342 (Invitrogen) for 10 mins. Embryos and oocytes were placed in PBS ready for imaging. All aqueous stages with embryo and oocyte staining took place under mineral oil (Sigma) to prevent excessive evaporation of solutions. Confocal images were acquired using an LSM510meta confocal microscope (Carl Zeiss MicroImaging, Inc) and analysed using LSM software. All positive samples were corrected by normalisation against control sample background levels.

2.2.12 ES cell culture and differentiation

ES cell culture was performed in the laboratory of Valerie Kouskoff (Paterson Institute, Manchester). ES cells were cultured on irradiated embryonic feeder cells (EFC) in Dulbecco's Modified Eagle Medium (DMEM) with 15% foetal calf serum (FCS), streptomycin, penicillin, leukaemia inhibitory factor (LIF; 1% conditioned medium) and 1.5×10^{-4} M monothioglycerol (MTG; Sigma) (Kouskoff 05, Fehling 03). 48 hours prior to differentiation, ES cells were transferred to gelatine coated plates in the same media. For the generation of

embryoid bodies (EBs), ES cells were trypsinized and plated at various densities in differentiation cultures. To generate erythrocyte lineages, EBs cells were placed in 60 mm Petri grade dishes in IMDM supplemented with 15% FCS, transferrin (200 μg/ml), 2 mM L-glutamine (Gibco/BRL), 0.5 mM ascorbic acid (Sigma), and $4.5x10^{-4}$ M MTG (Sigma). Cells were cultured at a density of $4x10^5$/ml in ultra low attachment 24-well plates (Costar). Cells were cultured in a humidified chamber in a 5% CO_2/air mixture at 37°C (Fehling, Lacaud et al. 2003; Kouskoff, Lacaud et al. 2005). Cells at days 0,1,4,5 and 6 of culture were either lysed using western blot lysis protocol as shown in 2.2.4 or prepared for RT-PCR as shown in 2.2.

CHAPTER 3

3 The S100A8 null mouse phenotype

3.1 Introduction

The S100 protein family members, S100A8 and S100A9, are abundant in adult myeloid cells where they form a stable heterodimer (Edgeworth, Gorman et al. 1991). Bone marrow is a good source of myeloid cells and is a good source of positive control cells for S100A8 and S100A9 protein. Many functions have been proposed for the two S100 proteins especially in neutrophils where the heterodimer forms 45% of cytosolic protein (Edgeworth, Gorman et al. 1991). To investigate the function of S100A8 and S100A9 mouse models were generated lacking either S100A8 or S100A9. S100A9 null mice were viable and showed no gross abnormalities (Hobbs, May et al. 2003). It was of interest that S100A9 null myeloid cells lacked S100A8 protein but not S100A8 mRNA (Hobbs, May et al. 2003). Throughout this thesis the S100A9 null mouse is frequently used as a model for studying the S100A8 null phenotype. There are distinct advantages to this approach the most important being that in wildtype mice circulating myeloid cells contain abundant amounts of S100A8 and S100A9 heterodimer. This will inevitably contaminate tissue samples for techniques like western blot and RT-PCR amongst others. In the S100A9 null mouse circulating myeloid cells do not stably express S100A9 or S100A8 protein. This allows tissue samples from this model to be assessed for S100A8 protein expression free from myeloid cell contamination. As the S100A9 null mouse is viable and grossly normal it is assumed in these studies that this S100A8 and S100A9 protein expression is the only significant difference with wildtype.

The S100A9 null mouse was viable and exhibited no gross abnormalities so expectations were similar for the S100A8 null mouse. However, the S100A8 null mouse was reported as embryonic lethal at E 9.0 (Passey, Williams et al. 1999). The report states that this is due to lack of S100A8 expression in a subset of infiltrating cells from the ectoplacental cone at E 6.5-7.5. There were inconsistencies both within the report and with unpublished data from our own lab, which have not been clarified or expanded upon since this publication. Passey et al 1999 proposed a cause of lethality and time of lethality that were separated by almost 2 days of development. Only speculation is offered as to the function of the expression or why it takes 2 days before the S100A8 null embryos are lost. The expression of S100A8 mRNA is shown only at limited stages (E 7.5 – E 8.5) and the expression of S100A8 protein is not shown. This was a key issue as S100A9 null myeloid cells were positive for S100A8 mRNA but not S100A8 protein suggesting S100A8 protein needs stabilisation *in vivo*. It was also not stated which strain of mouse was used in the Passey et al 1999 report. This became clear only through personal communication. Our initial characterisation experiments agreed that the S100A8 null mouse was embryonic lethal. It was shown with Y-chromosome staining of male embryos that S100A8 mRNA expression in the decidua is of maternal origin and not foetal (Hobbs, May et al. 2003). This is an important observation as no embryonic lethal phenotype can be attributed to maternal expression. We could also not find any S100A8 null embryos after implantation (dissected embryos at E 7.5, 8.5 and 9.5 genotyped by M.Mathies).

S100A8 null lethality caused solely by cells from the ectoplacental cone region should be overcome through a technique called tetraploid aggregation. In this technique a chimera is formed between an early embryo and embryonic stem cells. The embryonic stem cells give rise to all trophoblast tissues and extra-embryonic tissues. If the cells causing a null lethality are of trophoblast or extra-embryonic origin then the tetraploid aggregation will rescue the mouse. We conducted a tetraploid aggregation experiment with S100A8 heterozygous crosses to generate blastocysts coupled with wildtype embryonic stem cells. We genotyped the resultant mice born and found that no S100A8 null mice were born. This showed that the S100A8 null lethality could

not be, exclusively, caused by cells of trophoblast or extra-embryonic origin. It is still possible that S100A8 could have a non-redundant function in these cells but it would have also to be involved in foetal tissues.

The different phenotypes displayed by the S100A9 and S100A8 null mice have been highlighted. The loss of S100A9 in myeloid cells prevents stable S100A8 protein expression. If S100A9 is always required to stabilise S100A8 then the S100A8 null mouse should have an identical phenotype to the S100A9 null mouse. The S100A8 null phenotype shows that there must be at least one situation in mouse development where S100A8 protein is expressed and stable, independently of S100A9 protein. This suggests different roles for S100A8 and S100A9 in development with S100A8 performing a critical role.

The inconsistencies between our experiments and the reported phenotype lead to the conclusion that a more thorough examination of the model would yield more information on the S100A8 null phenotype. It was believed that the role of S100A8 could be more complex than portrayed in Passey et al 1999 and could yield more details on the function of S100A8 and S100A9 in myeloid cells and epithelia. The S100A8 null mouse is the most severe and only lethal phenotype reported to date for an S100 family protein deletion. It is of considerable interest to know what the function of S100A8 in development is.

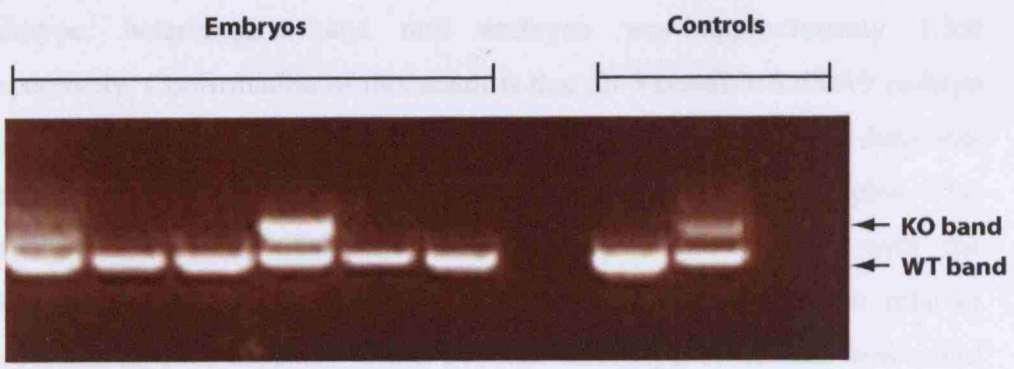
3.2 Results

3.2.1 No S100A8 null embryos are found postimplantation

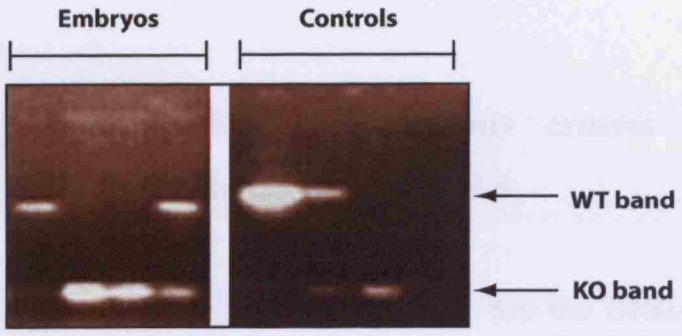
The Passey et al 1999 paper detailing the S100A8 null lethal phenotype presents evidence of genotyping of dissected embryos from S100A8 heterozygous crosses. A heterozygous cross should yield a 1:2:1 ratio of wildtype, heterozygous and null embryos respectively. The S100A8 null lethal phenotype should present as a loss of null embryos with no loss of wildtype or heterozygous embryos. The embryos were digested and using PCR the S100A8 genotype assessed. The evidence presented shows normal levels of S100A8 wildtype, heterozygous and null embryos at E 6.5-7.5. At E 8.5 there were lower levels than expected of S100A8 null embryos. At E 9.5 no S100A8 null embryos could be detected and it was concluded that S100A8 null embryos terminate at approximately E 7.5-8.5 although it takes a further 2 days for all embryos to resorb. This evidence was at odds with unpublished data from M.Mathies in our lab showing that no S100A8 null embryos could be detected postimplantation. It was decided to reassess the S100A8 heterozygous cross genotyping to determine whether there really was a discrepancy between the two sets of data. S100A9 heterozygous crosses were also analysed for S100A9 wildtype, heterozygous and null embryos. The S100A9 null phenotype is not embryonic lethal and so no loss of S100A9 null embryos should be seen. This is an elegant control for the genotyping of S100A8 heterozygous crosses because the S100A9 heterozygous control crosses will detect whether all possible genotypes can be cleanly determined. The results are shown in as shown in fig 3.1 and include highlighted data contributed by M.Mathies.

Embryos from S100A8 and S100A9 heterozygous crosses were dissected at E 7.0 and digested. The genotypes were analysed using the appropriate primers for S100A8 and S100A9. No S100A8 null embryos were found showing that the S100A8 lethality occurs prior to E 7.0 in our model. The ratio of S100A8

A



B



C

Strain	Stage	+/+	+/-	-/-	Total
S100A8	E 8.5	3	6	0	9*
S100A8#	E 7.5	9	22	0	31*
S100A8	E 7.0	5	10	0	15
S100A9	E 7.0	4	3	5	12

Fig 3.1 Genotyping of post-implantation embryos generated by S100A8 heterozygous crosses. PCR of disected embryos looking at the S100A8 genotypes from S100A8 heterozygous crosses (A) and the S100A9 genotypes from S100A9 heterozygous crosses (B) with genotyping results shown in table format (C). * = These data contributed by M.Mathies. #= Cross showed significant deviation from expected genotype ratios $p < 0.01$

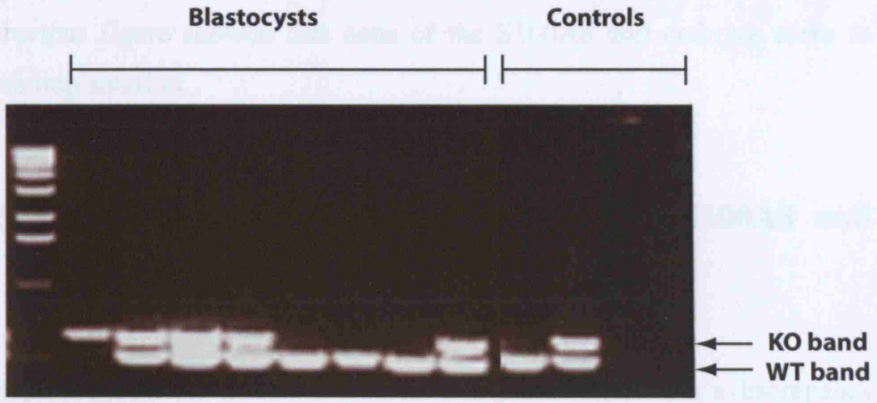
wildtype, heterozygous and null embryos was approximately 1:3:0 respectively. Confirmation of this result is that all 3 possible S100A9 embryo genotypes are clearly detected at the same embryonic stage, which discounts the possibility of maternal cell contamination of the embryo samples. This confirms the earlier observations of M.Mathies and conflict with the observations reported in Passey et al 1999. The increase in the relative proportion of heterozygotes in the S100A8 heterozygous crosses was noted although its significance is unknown. The S100A9 heterozygous crosses showed that S100A9 null embryos can be detected in this system.

3.2.2 Genotyping of S100A8 heterozygous crosses shows reduced S100A8 null blastocysts at E 4.5

Our previous experiment showed that our results for the S100A8 null phenotype differed from the reported phenotype in Passey et al 1999. In that report the level of S100A8 null blastocysts was shown as normal. Our data indicate that the S100A8 null lethality could occur earlier in development than previously thought. Attempts were made to genotype embryos earlier than E 6.5 but this proved to be technically too difficult and so it was decided to genotype S100A8 heterozygous crosses at the blastocyst stage as shown in fig 3.2. Genotyping the blastocysts would show whether S100A8 null embryos were present at normal levels prior to implantation. It would also provide another comparison between our observations and the reported S100A8 null phenotype.

E 4.5 blastocysts were generated from S100A8 heterozygous crosses, digested and genotyped using the 2-step PCR protocol detailed in chapter 2. The level of S100A8 null blastocysts was significantly reduced but they were seen. The results show S100A8 null blastocysts at approx 80% of expected levels from Mendelian ratios. This result was the first time S100A8 null embryos had been seen in our mouse model. The data indicate that the majority of S100A8 null embryos are lost by E 4.5 and suggests that the point of lethality for the S100A8 null mouse is earlier than E 4.5. The small number of S100A8

A



B

Genotype	+/+	+/-	-/-	Total
E 4.5 #	11	28	2	41

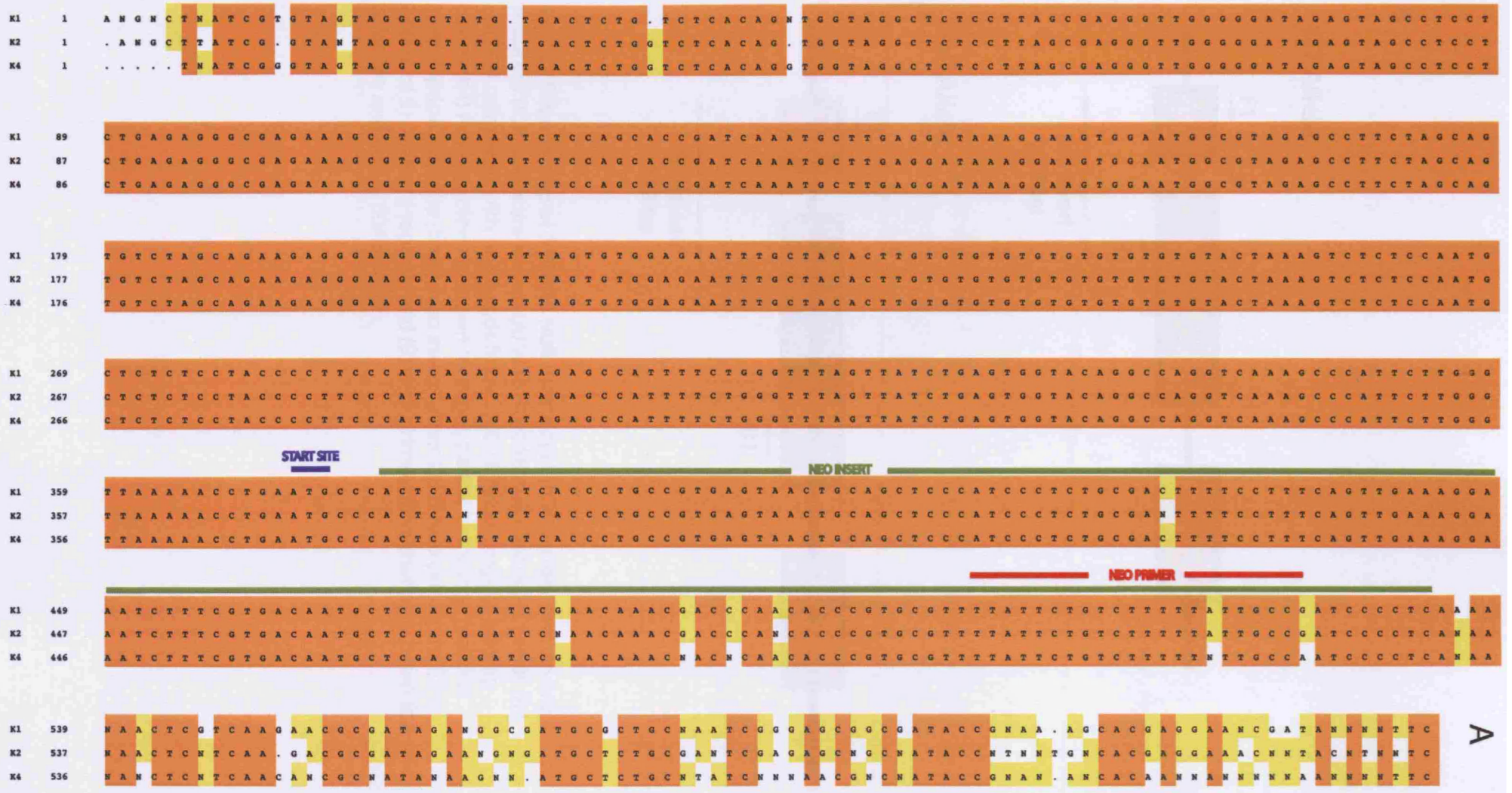
Fig 3.2 PCR of blastocysts generated from S100A8 heterozygous crosses. PCR genotyping E 4.5 blastocysts generated from S100A8 heterozygous crosses (A) with the results in table format (B). Cross showed significant deviation from expected genotype ratios $p < 0.01$.

null embryos that survive to E 4.5 are of interest although it must be noted that the previous figure showed that none of the S100A8 null embryos seem to survive implantation.

3.2.3 Sequencing of PCR bands confirms the S100A8 null embryos

The evidence from our genotyping studies suggests that there is a discrepancy between our observations and the reported phenotype of the S100A8 null phenotype. It is important to verify that the differences in the observations are caused by the S100A8 gene and not due to problems with the genotyping evidence or with mouse strain effects. There was a concern that the bands seen in blastocyst PCR might not correlate with S100A8 null bands. Sequencing of the PCR bands would confirm that we are indeed looking at the effects of the S100A8 gene. This is of help especially where we have used multiple step PCR processes. The S100A8 gene silencing produced a null allele that is easy to distinguish due to the presence of a NEO cassette inserted into the second exon of the S100A8 gene. Sequencing of the S100A8 null bands from PCR gels should show the S100A8 gene sequence with a correctly inserted NEO cassette. The null band primers will produce a section of DNA with 468bp of S100A8 gene followed by the Neo cassette sequence. The results of sequencing are shown in fig 3.3.

S100A8 null bands were analysed from two previously genotyped E 4.5 S100A8 null embryos after the 2-step PCR protocol. The bands were compared to the null band from known S100A8 heterozygous mouse tailsnip, which is processed after the one step S100A8 PCR protocol. S100A8 null PCR bands were cut from agarose gels, digested and sequenced. Sequencing showed that the S100A8 null bands from both the blastocyst PCR bands were identical to the null band from the control S100A8 heterozygous tail snip sample. The sequenced bands show the correct part of the S100A8 gene along with the correctly positioned NEO cassette site from homologous recombination used in gene silencing. Homology is not 100% but this is



A

B

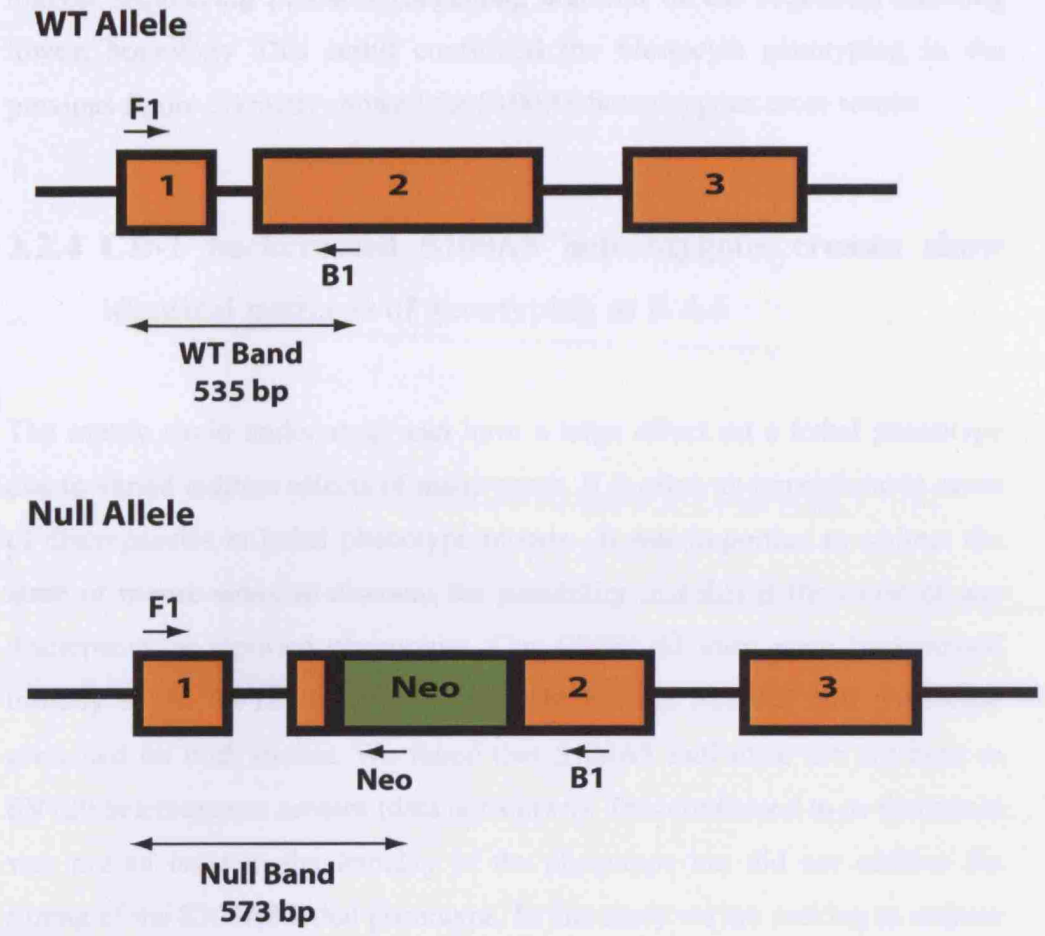


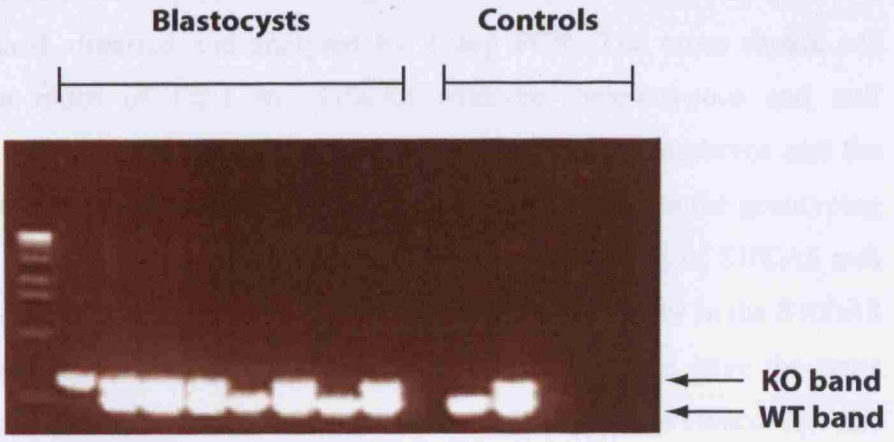
Fig 3.3 Sequencing of S100A8 null blastocyst PCR bands. Sequencing results of PCR bands are shown (A) with S100A8 null blastocyst bands (K1 and K2) compared with null bands from S100A8 heterozygous tailsnip bands (K4) Full homology is shown (orange), partial homology (yellow) transcription start site (blue), Neo insert (green) and Neo primer (red). Alleles of S100A8 are represented (B) with primer combinations shown for wildtype and null S100A8 bands.

considered normal within the sequencing technique used with the shortest and longest sequencing products (beginning and end of the sequence) showing lowest homology This result confirmed the blastocyst genotyping in the previous figure correctly showed the S100A8 heterozygous cross results.

3.2.4 CD-1 backcrossed S100A8 heterozygous crosses show identical patterns of genotyping at E 4.5

The mouse strain under study can have a large effect on a lethal phenotype due to varied indirect effects of many genes. It is often an unpredictable cause of discrepancies in lethal phenotype reports. It was important to address the issue of mouse strain to discount the possibility that this is the cause of any discrepancy in reported phenotypes. Our C57BL/6J mice were backcrossed initially to the SV129 strain to check whether the S100A8 null phenotype presented on both strains. We found that S100A8 null mice are not born to SV129 heterozygous crosses (data not shown). This confirmed to us that strain was not an issue in the lethality of the phenotype but did not address the timing of the S100A8 lethal phenotype. In this study we are seeking to address the issue of discrepancies in our observations and those previously reported and to do so we must cross our mice onto the same background used in Passey et al 1999. Our C57BL/6J mice were backcrossed against the CD-1 background used in the S100A8 null phenotype report (information from personal communication). The CD-1 strain is also a highly outbred mouse line and it was hoped that such a backcross would show whether genetic background was a factor in the S100A8 null phenotype. It would be expected that crossing our C57Bl/6J mice onto the CD-1 line will either confirm the findings in our mouse strain or show the same result seen in Passey et al 1999 - normal levels of S100A8 null blastocysts. The blastocyst stage was chosen, as there was a distinct difference between our data and the reported S100A8 null lethality at this stage. It was a possibility that the CD-1 backcross could give a novel third phenotype and therefore the blastocyst stage was deemed early enough to measure any potential outcome. The results are shown in fig 3.4.

A



B

Genotype	+/+	+/-	-/-	Total
E 4.5#	11	33	1	45

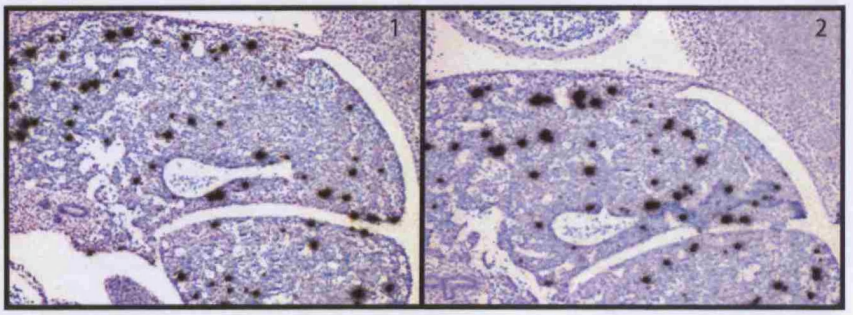
Fig 3.4 PCR of blastocysts generated from CD-1 backcrossed S100A8 heterozygous crosses. CD-1 backcrossed blastocyst genotypes from S100A8 heterozygous crosses were analysed (A) and the results presented in table format (B). Cross showed significant deviation from expected genotype ratios $p < 0.01$

CD-1 backcrossed S100A8 heterozygous crosses produced blastocysts that were flushed, digested and analysed by 2-step PCR. The cross should see Mendelian ratios of 1:2:1 for S100A8 wildtype, heterozygous and null embryos. Instead we see a 90% reduction in S100A8 null embryos and the ratio is closer to 1:3:0. The result is approximately the same as the genotyping results in our C57BL/6J mice in that there is a significant loss of S100A8 null embryos. The data show that genetic background is not a factor in the S100A8 null phenotype, as it seemed that highly outbred CD-1 mice gave the same result for S100A8 as our C57BL/6J strain. It is further evidence that the S100A8 null lethal phenotype occurs prior to E 4.5. Another notable point is that in the CD-1 strain as well as the C57BL/6J strain there is an increase in the levels of heterozygous embryos from the heterozygous crosses.

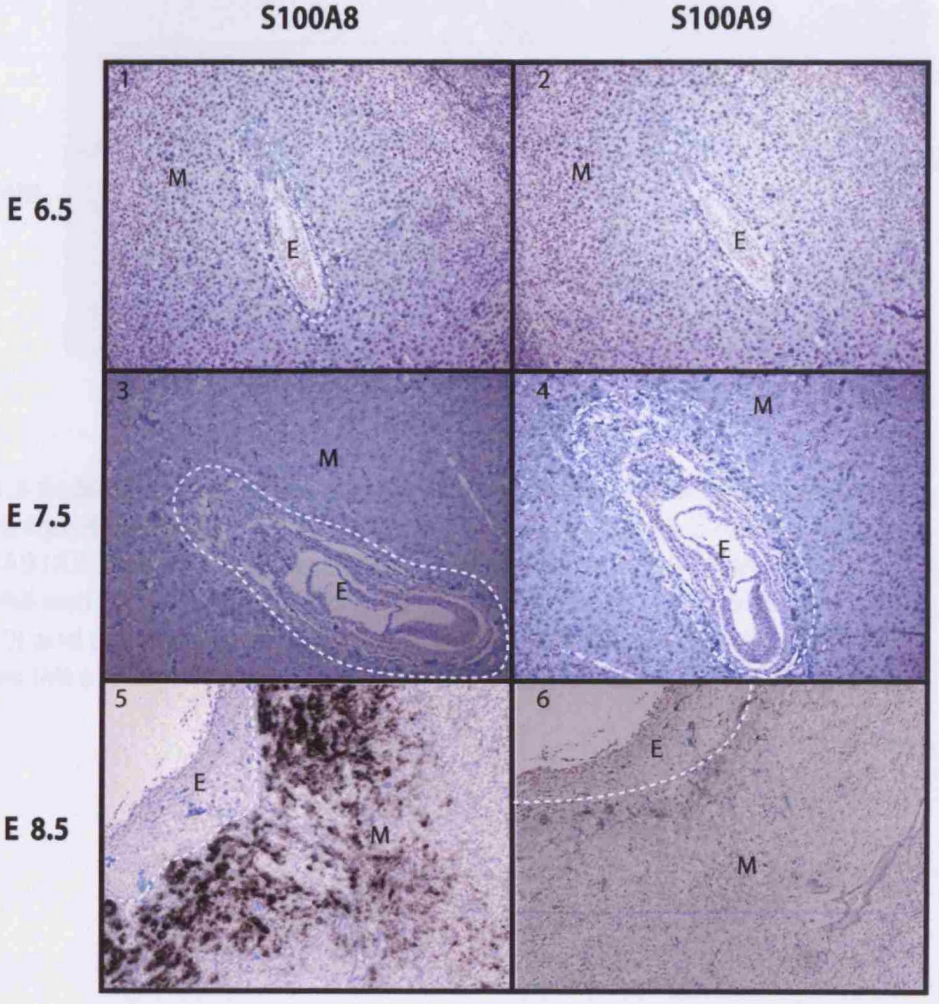
3.2.5 S100A8 mRNA expression occurs between E 7.5 and E 14.5 in the maternal decidua.

It was reported in Passey et al 1999 that S100A8 mRNA expression is seen in embryonic cells infiltrating the maternal decidua from the ectoplacental cone at E 7.0. To explain the S100A8 null lethal phenotype the cells are proposed to have a non-redundant function. Conflicting evidence is presented as to the origin of the S100A8 mRNA expression in Passey at al 1999 and Hobbs et al 2003. The former report states that the expression is embryonic while the later maintains that the expression is maternal. As the data and its interpretation are important in the analysis of the S100A8 genotype and in light of other discrepancies in our S100A8 null model it was decided to reassess this mRNA expression. Expression of S100A8 and S100A9 through this period could provide further evidence as to which tissue expresses S100A8 mRNA and whether this is indeed linked to the S100A8 null phenotype. The expression pattern would be analysed from implantation through to birth. In order to investigate the expression of S100A8 and S100A9 mRNA in development *in situ* hybridisation was used as shown in fig 3.5.

A



B



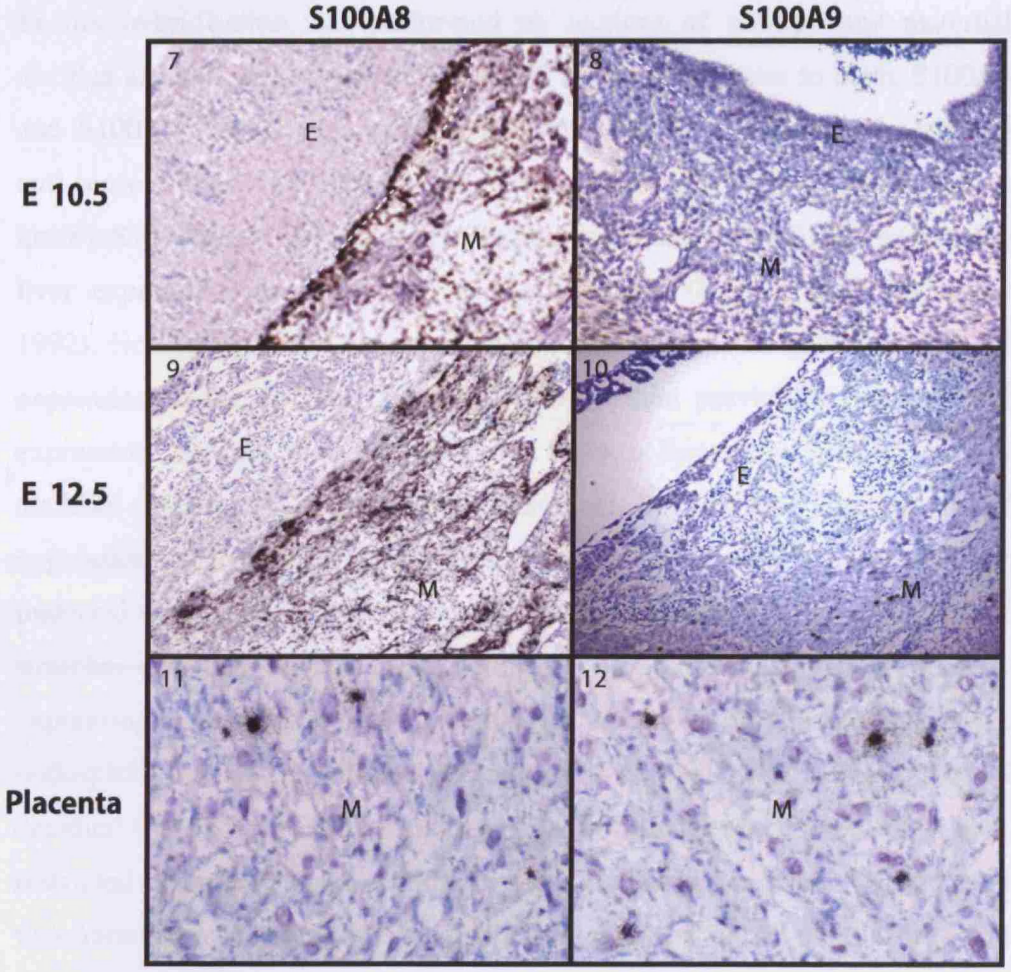


Fig 3.5 S100A8 and S100A9 expression in maternal decidua shown by *in situ* hybridisation. E 12.5 embryonic liver probed with S100A8 (A1) and S100A9 (A2) mRNA probes as positve control for decidual expression of S100A8 and S100A9 (B) at E 6.5 (B1,2) 7.5 (B3,4) 8.5 (B5,6) 10.5 (B7,8) 12.5 (B9,10) and placenta at birth (B11,12). Embryonic tissues (E) and matrnal tissues (M) are highlighted. Dashed white lines - maternal-foetal boundary.

In situ hybridisation was performed on sections of embryo and maternal decidua at E 6.5, 7.0, 8.5, 10.5, 11.5, 14.5 and placenta prior to birth. S100A8 and S100A9 mRNA expression is probed using P32 radioactively labelled anti-sense probes for S100A8 and S100A9 mRNA. As a positive control known S100A8 and S100A9 mRNA expression is shown by E 12.5 embryonic liver expression consistent with previous reports (Lagasse and Weissman 1992). No S100A8 mRNA expression can be seen at E 6.5 or E 7.5. S100A8 expression in the decidua is more prolonged than previously thought with expression detected at E 8.5, 10.5, 12. E 14.5. Expression can be seen in decidual tissues, is extensive yet has clear boundaries. The pattern of expression shows that the cells expressing the S100A8 mRNA are most likely maternal due to the widespread nature of the expression and the fact that it stretches deep into the maternal decidua. The possibility of S100A8 mRNA expressing infiltrating cells seems unlikely although it could be masked by the widespread maternal expression. Expression of S100A9 mRNA is also seen in decidual tissues from E 8.5 through to E 14.5 although the expression is very restricted compared with S100A8 mRNA. Throughout this E 8.5-14.5 timeframe a pronounced difference in expression of S100A8 and S100A9 can be seen. As previously shown (Hobbs, May et al. 2003) S100A8 expression is more widespread and abundant in maternal decidua than S100A9 expression. This difference in S100A8 and S100A9 is true at all stages through to E 14.5. In placenta prior to birth S100A8 and S100A9 mRNA expression have a similar and less extensive pattern suggesting that the difference in expression in earlier stages no longer applies.

3.2.6 S100A8 mRNA expression in maternal decidua is localised to vasculature

A detailed study of the S100A8 mRNA expression in maternal and embryonic tissues is important to analyse. The expression of S100A8 mRNA has a distinct boundary and appears to be in maternal decidual tissue. The maternal foetal boundary is marked by the embryo derived trophoblast giant cells, which are distinct in appearance and form a discrete layer, which can be

used to determine embryonic and maternal tissues. To see whether expression of S100A8 mRNA is maternal, foetal or both detailed analysis was undertaken as shown in fig 3.6. Comparison of S100A8 and S100A9 mRNA expression will also show whether there is any overlap in expression in the decidua or whether S100A8 mRNA is expressed differentially.

Expression of S100A8 mRNA is clearly observed in maternal decidual tissue and not foetal tissue. Analysis of the foetal maternal boundary, delineated by trophoblast giant cells, shows S100A8 expression on the maternal side. S100A8 mRNA expression is not seen in the trophoblast cell layer or any cells on the foetal side of this layer. S100A8 expression at all stages is on the maternal side of the trophoblast giant cell layer. Staining also shows that S100A8 expression is particularly strong around the vasculature in maternal decidua. This suggests possible endothelial cell expression or a sub-endothelial cell type. It should be noted though that in a highly vascular tissue like decidua most cells are within proximity of the vasculature. In addition to this the vast majority of cells in the decidua are positive for S100A8 mRNA showing that the decidual cells themselves are positive. Overlap analysis of S100A8 and S100A9 mRNA expression clearly shows that there is a population of both S100A8 and S100A9 mRNA expressing cells in the decidua. This expression is close to the trophoblast giant cell layer and does not extend deeply into the maternal decidua. This population could be neutrophils, which have been shown to localise to the foetal maternal boundary to clear apoptotic cells (McMaster, Dey et al. 1993). This S100A8 and S100A9 mRNA expressing population is small compared to the S100A8 only expressing maternal decidual cells. S100A8 mRNA expression extends deeply into the maternal decidua and it seems clear that this is a different population. The data show that there are two S100A8 mRNA expressing populations in the maternal decidua.

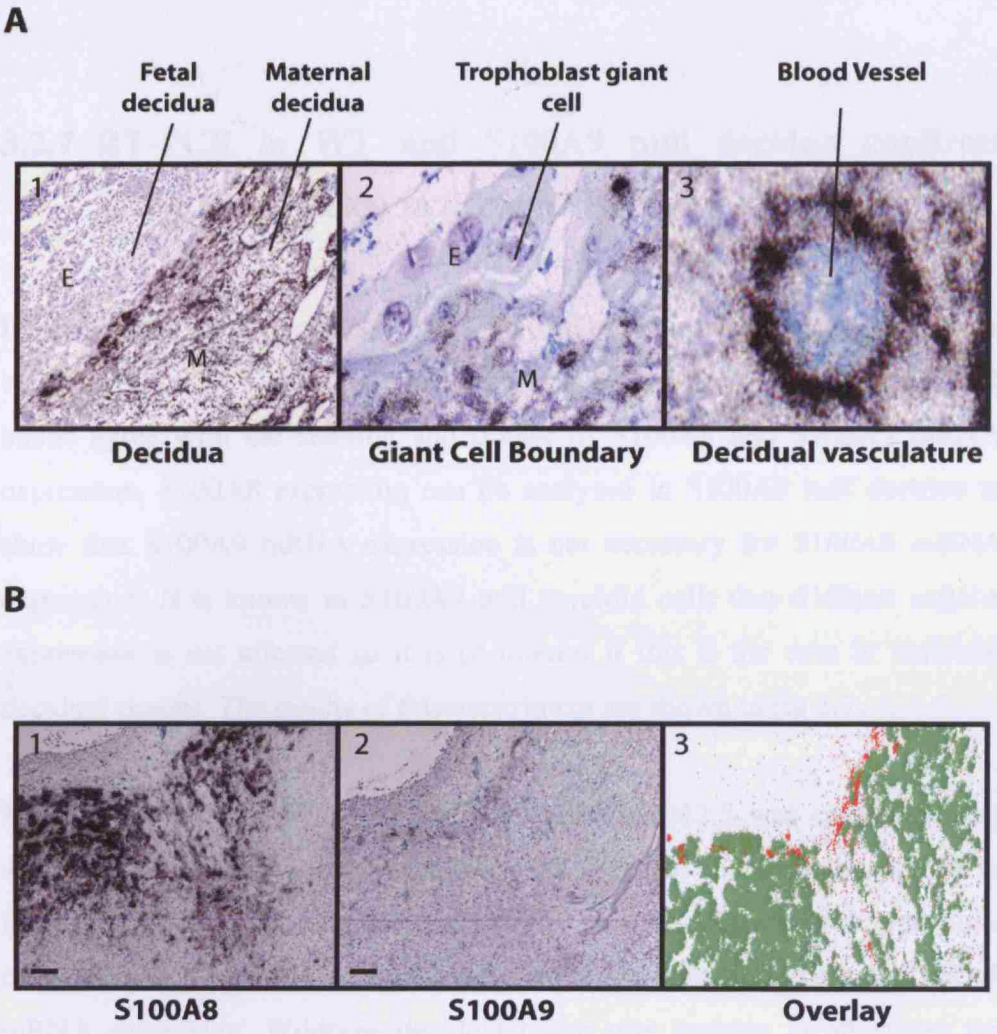


Fig 3.6 Expression of S100A8 mRNA is confined to maternal decidua and is more extensive than expression of S100A9 mRNA. Maternal S100A8 mRNA expression has a distinct boundary in development shown here at E 12.5 (A1), which is delineated by the Trophoblast Giant cells of the embryo (A2). Within the decidual tissue staining is highly localised to the vasculature (A3). Comparison of S100A8 and S100A9 expression at E 8.5 (B1,2) shows overlap of S100A8 and S100A9 expression at the trophoblast giant cell boundary (B3). Expression of S100A8 alone is seen throughout the surrounding decidal tissue. M- maternal E-embryonic. Green- S100A8 Red- S100A9

3.2.7 RT-PCR in WT and S100A9 null decidua confirms S100A8 expression in decidual tissues

Expression of S100A8 and S100A9 by *in situ* hybridisation must be confirmed by using RT-PCR of the tissues to see whether the S100A8 and S100A9 PCR bands agree with the staining and timing of S100A8 and S100A9 mRNA expression. S100A8 expression can be analysed in S100A9 null decidua to show that S100A9 mRNA expression is not necessary for S100A8 mRNA expression. It is known in S100A9 null myeloid cells that S100A8 mRNA expression is not affected so it is of interest if this is the case in maternal decidual tissues. The results of this experiment are shown in fig 3.7.

Maternal decidual tissue at stages E 8.5, 10.5 and 12.5 was extracted from wildtype and S100A9 null implantation sites, digested and analysed using RT-PCR for expression of S100A8 and S100A9 mRNA. Digested wildtype bone marrow cells were used as a positive control for both S100A8 and S100A9 mRNA expression. Wildtype decidual tissue was positive at all stages for S100A8 and S100A9 mRNA expression, which reflects the *in situ* mRNA expression data. S100A9 null decidual tissue samples are all positive for S100A8 mRNA expression. As expected all S100A9 null decidual samples were negative for S100A9 mRNA expression. The RT-PCR result does not show relative amounts of mRNA expression, which is more clearly shown by *in situ* staining in fig 3.5.

3.2.8 Neutrophils are the small S100A8 and S100A9 mRNA expressing population in maternal decidua.

Expression of high levels of S100A8 and S100A9 mRNA is a distinct marker for neutrophils. It was highly likely that the S100A8 and S100A9 mRNA expressing population in the maternal decidua were neutrophils. The expression patterns were also consistent with previous reports of neutrophil

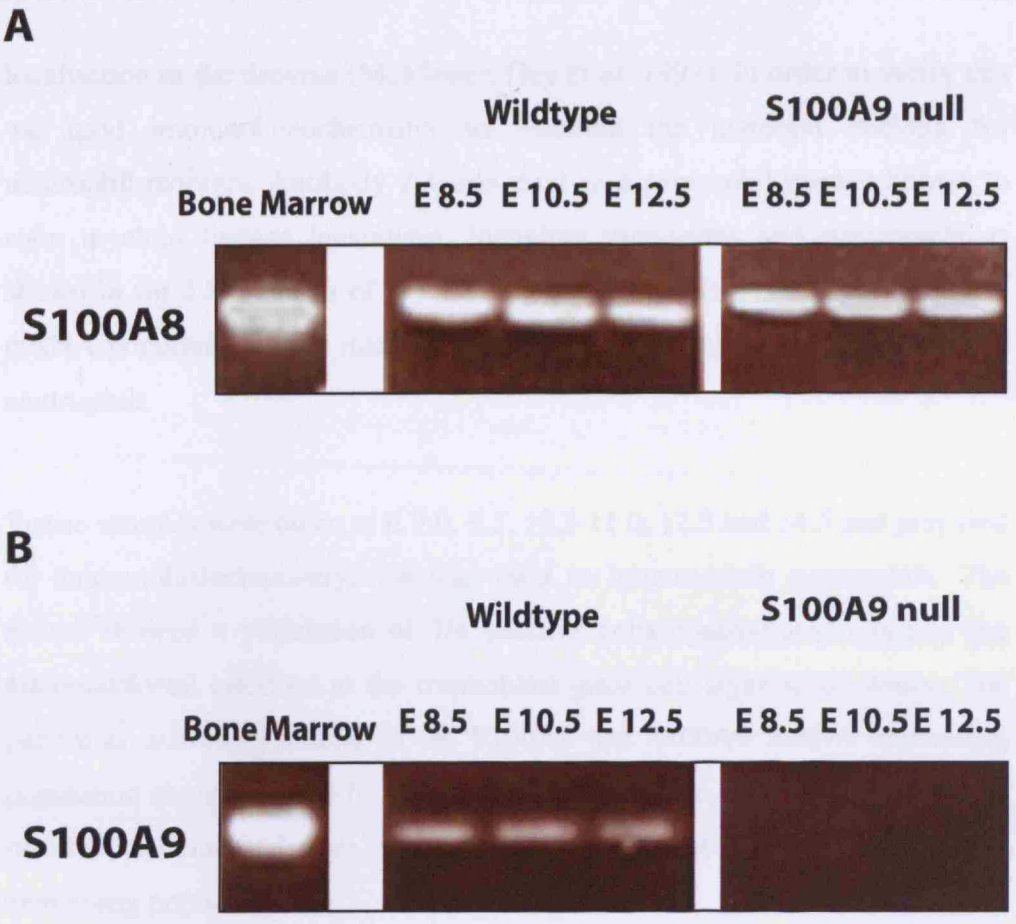


Fig 3.7 RT-PCR of maternal decidual tissue. RT-PCR of decidual tissue from wildtype and S100A9 null mice at E 8.5, 10.5 and 12.5. Expression of S100A8 mRNA (A) and S100A9 mRNA (B) are shown.

localisation in the decidua (McMaster, Dey et al. 1993). In order to verify this we used immunohistochemistry to examine the maternal decidua for neutrophil markers. Antibody 7/4 was used as a neutrophil marker known to stain myeloid lineage leukocytes, including monocytes and neutrophils as shown in fig 3.8. Patterns of 7/4 staining similar to the S100A8 and S100A9 mRNA population in the maternal decidua should confirm the population as neutrophils.

Tissue samples were taken at E 7.0, 8.5, 10.5 11.0, 12.5 and 14.5 and prepared for immunohistochemistry. 7/4 was used to immunostain neutrophils. The results showed a population of 7/4 positive cells concentrated close to the maternal/foetal interface at the trophoblast giant cell layer at all stages. The pattern is extremely similar to the S100A8 and S100A9 mRNA expressing population shown in fig 3.6. The pattern of expression is not extensive in the maternal decidua and does not show similarity to the S100A8 mRNA only expressing population.

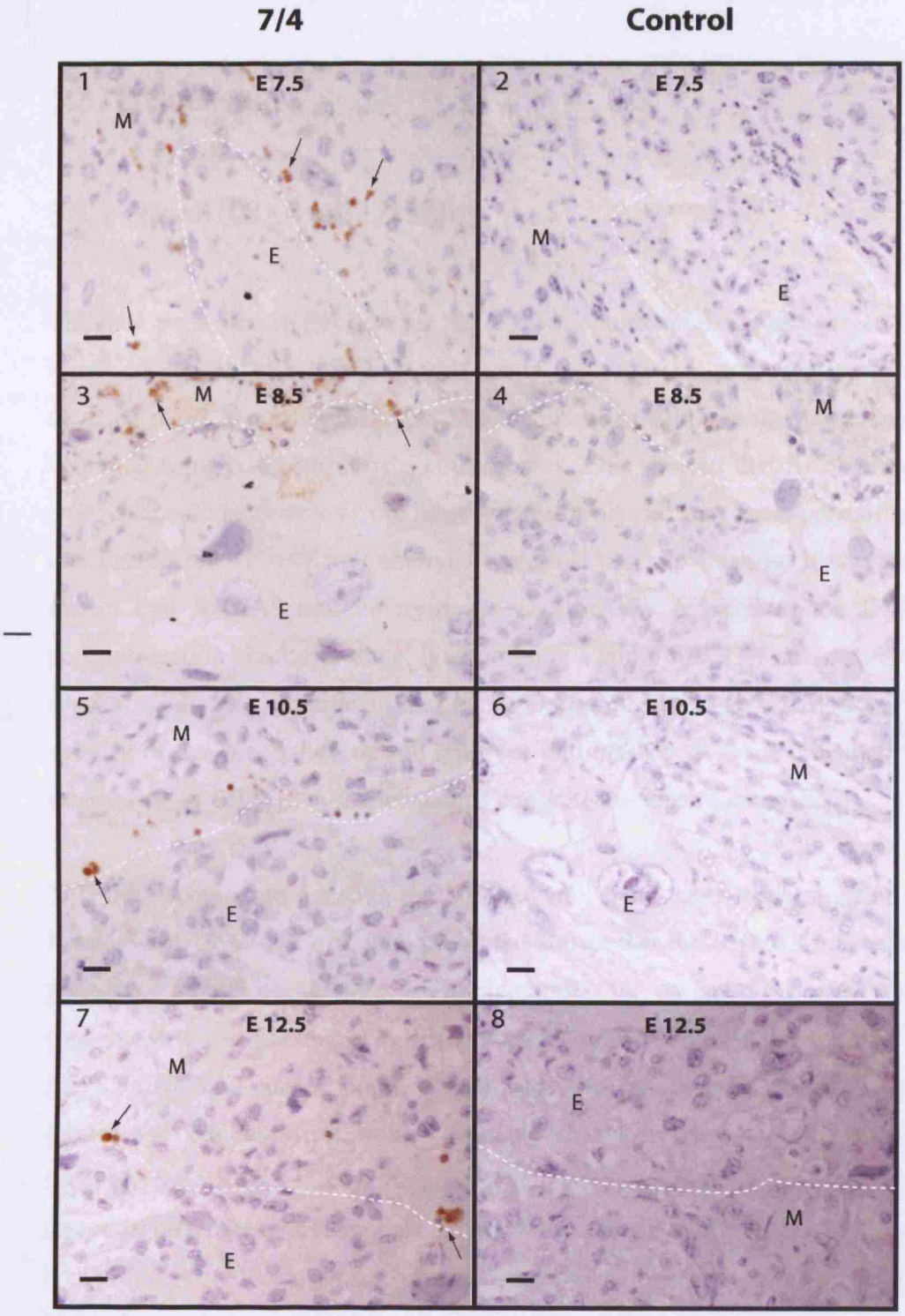


Fig 3.8 7/4 staining in maternal decidua. Immunohistochemistry showing 7/4 staining at E 7.5 (A1-3) E 8.5 (B1) E 10.5 (B3) E 12.5 (B5). Controls for the stages are also shown (B2,4,6). 7/4 staining using 7/4 Ab followed by anti-rabbit secondary. Positive 7/4 staining shown in brown. M - maternal, E - embryo. Dashed white line -foetal-maternal interface. Scale bar = 20 μM.

3.3 Discussion

3.3.1 The S100A8 null lethality

The data presented in this chapter has shown significant differences from the report of Passey et al 1999. In this study S100A8 null embryos were not detected by PCR postimplantation. The technique of genotyping embryos was validated using S100A9 heterozygous crosses. This showed that S100A8 null embryos could be detected from heterozygous crosses if they were present and that indeed the S100A8 null embryos were lost postimplantation. It was then shown that S100A8 null embryos are significantly reduced at the E 4.5 preimplantation blastocyst stage. Backcrossing against the CD-1 strain showed similar results with significant loss of S100A8 null blastocysts at E 4.5. The validity of the genotyping of null embryos and blastocysts was confirmed by sequencing of S100A8 null allele bands compared to known controls.

This data brings into question the S100A8 null lethal phenotype reported in Passey et al 1999. The evidence presented shows that the S100A8 null lethal phenotype could occur prior to implantation, far earlier than previously thought. It is still possible that differences in mouse strain or the creation of the S100A8 null mouse could account for the discrepancies between our model and the report. It is also possible that there are multiple distinct functions for S100A8 in development, which could cause lethality at different stages in different mouse models. If this were the case differences in mouse background could account for different stages of lethality in separate models. If this is not the case then the phenotype of the S100A8 null mouse could be very different from the published report and shed new light on S100A8 function in development.

Embryonic lethality prior to implantation in the S100A8 null mouse would be of considerable interest and would provide vital insight into the function of the protein. Embryonic development prior to implantation is a strictly controlled

sequence of events, which will involve distinct developmental pathways and control mechanisms. It can be postulated that S100A8 will be involved in these developmental processes. It could be that S100A8 null embryos will present a distinct preimplantation phenotype, which can be connected to a known developmental process. However, the nature of the genotyping results in fig 3.2 and fig 3.4 suggest that this may not be the case. The small number of S100A8 null embryos that survive to blastocysts at E 4.5 in both C57BL/6J and CD-1 crosses implies that the S100A8 null embryos do not die immediately upon encountering the S100A8 dependent function but may die slowly thereafter. It might be possible to observe either an abrupt loss or slow death of S100A8 null embryos by culturing embryos *in vitro*. Such observations could also indicate the exact timing of S100A8 null lethality.

3.3.2 Maternal decidual expression of S100A8 mRNA

It has been shown that there is extensive expression of S100A8 mRNA in maternal decidua between E 7.5 and E 14.5 occurring in two distinct populations. The data was consistent with previous reports but showed a more prolonged expression of S100A8 mRNA than previously thought. One population of S100A8 and S100A9 mRNA expressing cells is in all probability neutrophils. The expression of both S100A8 and S100A9 was a distinct marker for neutrophils and the vascular area close to the trophoblast giant cell layer should contain circulating neutrophils. It is known that neutrophils are restricted to the foetal maternal boundary where necrotic cells need clearing (McMaster, Dey et al. 1993; Fernekorn and Kruse 2005). The further confirmation of positive 7/4 staining reinforces the identity of this population. The pattern of S100A8 mRNA, S100A9 mRNA and 7/4 is highly correlated in this population The second population of S100A8 mRNA-only expressing cells is a population resident in the maternal decidua throughout development to E 14.5 and certainly includes mesometrial decidual cells. It is not clear if this population is present in virgin endometrium or whether it is a population resulting from the decidual reaction postimplantation. At 7.0 the mesometrium begins to differentiate (Abrahamsohn and Zorn 1993) and from

the literature it seems certain that expression of S100A8 mRNA occurs in differentiating mesometrium. Whether it is a resident cell type or a product of differentiation then it is of interest to discover what the trigger for S100A8 mRNA expression is. The implantation of an embryo into maternal decidua is a specialised immune reaction in that the embryo is genetically distinct from the mother. With this in mind the cells expressing S100A8 mRNA could include immune cells responding to this specialised inflammatory situation. If this is the case the timeframe of S100A8 mRNA expression could reflect the profile of immune cell recruitment to the decidua. It is of interest why S100A8 expression only occurs 48 hours after implantation although this is possibly due to differentiation within the decidua.

The pattern of S100A8 mRNA expression did not extend to the embryonic contribution to the placenta. Specifically no S100A8 mRNA was seen at E 6.5 or E 7.0 as was reported in Passey et al 1999. Embryonic S100A8 mRNA expression is an essential requirement to demonstrate an embryonic lethal phenotype for the S100A8 null embryo yet was not seen in this postimplantation study. Our finding was supported by the lack of S100A8 null embryos identified in postimplantation studies in fig 3.1. The possibility of embryonic expression postimplantation in wildtype cannot conclusively be ruled out but the evidence of widespread and continued S100A8 mRNA expression in the maternal tissues suggests that there is clearly a maternal expression and casts doubt upon any embryonic expression. With such high S100A8 mRNA expression in the maternal decidua it is possible to misread a possible embryonic expression, which may have occurred in Passey et al 1999. The report of infiltrating cells expressing S100A8 mRNA was further undermined by the existence of 7/4 staining cells close to the EPC at E 7.0. 7/4 expressing neutrophils or monocytes both express S100A8 mRNA and could have caused confusion and incorrectly attributed to "infiltrating" embryonic cells.

Existence of a cell type that expresses S100A8 mRNA but not S100A9 mRNA is of interest for two reasons. It may point to the existence of an *in vivo* situation where S100A8 protein is expressed that is not stabilised by

S100A9 protein. In the S100A9 null myeloid cells S100A8 protein is not stable without S100A9 protein. In has been widely shown that S100A8 can form stable homodimers and multimers *in vitro* under many conditions. Many roles have been assigned to such S100A8 complexes *in vitro* including anti-oxidant activity (Harrison, Raftery et al. 1999) and chemoattraction (Lackmann, Cornish et al. 1992). All of this work has been conducted *in vitro* and as yet few *in vivo* studies have supported an *in vivo* role for S100A8 homodimers. It has been proposed that S100A8 homodimers are not energetically favourable as they lack appropriate complementary residues in the dimerisation plane (Hunter and Chazin 1998). A cell type that expresses S100A8 mRNA independently of S100A9 mRNA could therefore provide an *in vivo* source of S100A8 multimers. It is also possible that S100A8 can bind an as yet unidentified alternative binding partner. A putative S100A8 partner could provide insight into the role of S100A8 in the maternal decidua or indicate a specialised role distinct from the function of the S100A8/S100A9 heterodimer in myeloid cells. Our data show a potential role for S100A8 in maternal decidual tissue postimplantation. This role is not clear but it is clear that any role is highly unlikely to explain the S100A8 null phenotype.

3.3.3 Conclusions and further study

Two general conclusions can be reached from the data presented in this chapter. The S100A8 null phenotype is more complex than originally thought. There is evidence of a role in development for S100A8 between E 7.5 and E 14.5 but this is restricted to the maternal decidua. This role is of interest despite being highly unlikely to be linked to the S100A8 null phenotype. This role is analysed in chapter 4. There is also evidence that the S100A8 null phenotype is a preimplantation lethality. This could show insight to the function of S100A8 in development and will be addressed in chapter 5.

CHAPTER 4

4 S100A8 in postimplantation development

4.1 Introduction

Having established grounds for re-examining the S100A8 null phenotype, two avenues of investigation were possible. One strand of questioning was whether S100A8 has a role in postimplantation development. There is undisputable evidence of S100A8 mRNA expression in maternal decidua, where the function is unknown. S100A8 may have a redundant role in postimplantation development and a non-redundant role in preimplantation development. It may be that any postimplantation role for S100A8 will provide evidence and insight into a preimplantation role for S100A8. This chapter examines the postimplantation role of S100A8 and seeks to answer the questions as to its function in maternal and embryonic tissues.

Many of the tissues and cells covered in this chapter are introduced in chapter 1 with a summary presented here. Haematopoiesis in the developing embryo begins in the yolk sac at E 7.0 with the generation of primitive erythrocytes in the yolk sac blood islands (Moore and Metcalf 1970). By E 8.5 the primitive myeloerythroid precursors arise that develop into definitive erythrocytes and macrophages (Cumano, Dieterlen-Lievre et al. 1996). Placenta is also believed to be an active haematopoietic tissue at E 9.0 (Alvarez-Silva, Belo-Diabangouaya et al. 2003). At E 10.5 haematopoietic ES cells (HSCs) arise in the intra-embryonic aorta-gonad-mesonephros (AGM) region (Medvinsky, Samoylina et al. 1993). HSCs can also be seen at E 10.5 in the placenta and yolk sac although the origin and potency of these cells is unclear. Haematopoiesis develops through a series of migrations from initial

haematopoietic tissues of yolk sac placenta and AGM to the foetal liver (Cumano and Godin 2007). Liver development begins in the definitive endoderm at E 8.5. Fibroblast growth factor (FGF) and bone morphogenic protein (BMP) signalling from nearby tissues induce hepatic gene expression in the ventral foregut wall (Jung, Zheng et al. 1999; Rossi, Dunn et al. 2001). The hepatic endodermal cells proliferate and, with the assistance of endothelial cells from the mesoderm, form a liver "bud". The liver bud emerges from the developing gut tube and can be colonised by haematopoietic cells as early as E 9.5.

The colonising cells, primarily myeloerythrocyte precursors, arrive from the yolk sac as circulation begins. At E 11.5 HSCs can be seen in the foetal liver and are believed to migrate there from the AGM, yolk sac and placenta as the onset of circulation allows. The foetal liver is a more mature haematopoietic site than yolk sac with more cell types arising from the multipotent HSC whose population expands within the liver. Previous reports examining S100A8 and S100A9 in development have concentrated on myeloid cell differentiation in the foetal liver. It is well documented that S100A8 and S100A9 are expressed in myeloid cells, which first arise in the foetal liver at E 12.5 (Lagasse and Weissman 1992). At this stage S100A8 and S100A9 positive cells resembling polymorphonuclear neutrophils can clearly be seen (Lagasse and Weissman 1992). This was thought to be the first expression of S100A8 and S100A9 in development although as the studies were concentrated on myeloid differentiation no study had specifically looked earlier in development than E 12.5 before the work of Passey et al 1999.

In vitro systems using embryonic stem (ES) cells generated from embryoid bodies have proved a useful model for haematopoietic development especially in yolk sac tissues (Keller, Kennedy et al. 1993). Many of the genes and signalling processes involved in lineage differentiation show significant similarities between *in vivo* tissues and cultured cells (Kouskoff, Lacaud et al. 2005). This is especially true of early haematopoietic events such as the generation of haemangioblasts (Kouskoff, Lacaud et al. 2005), which can be difficult to study *in vivo*. The similarity of molecular events in ES

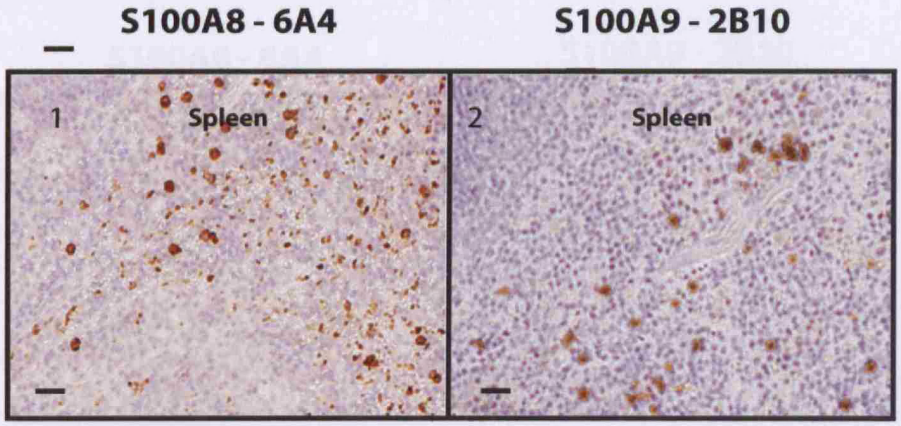
cell derived haematopoiesis makes it a good *in vitro* model to determine which proteins are expressed and play a role in this process *in vivo*.

4.2 Results

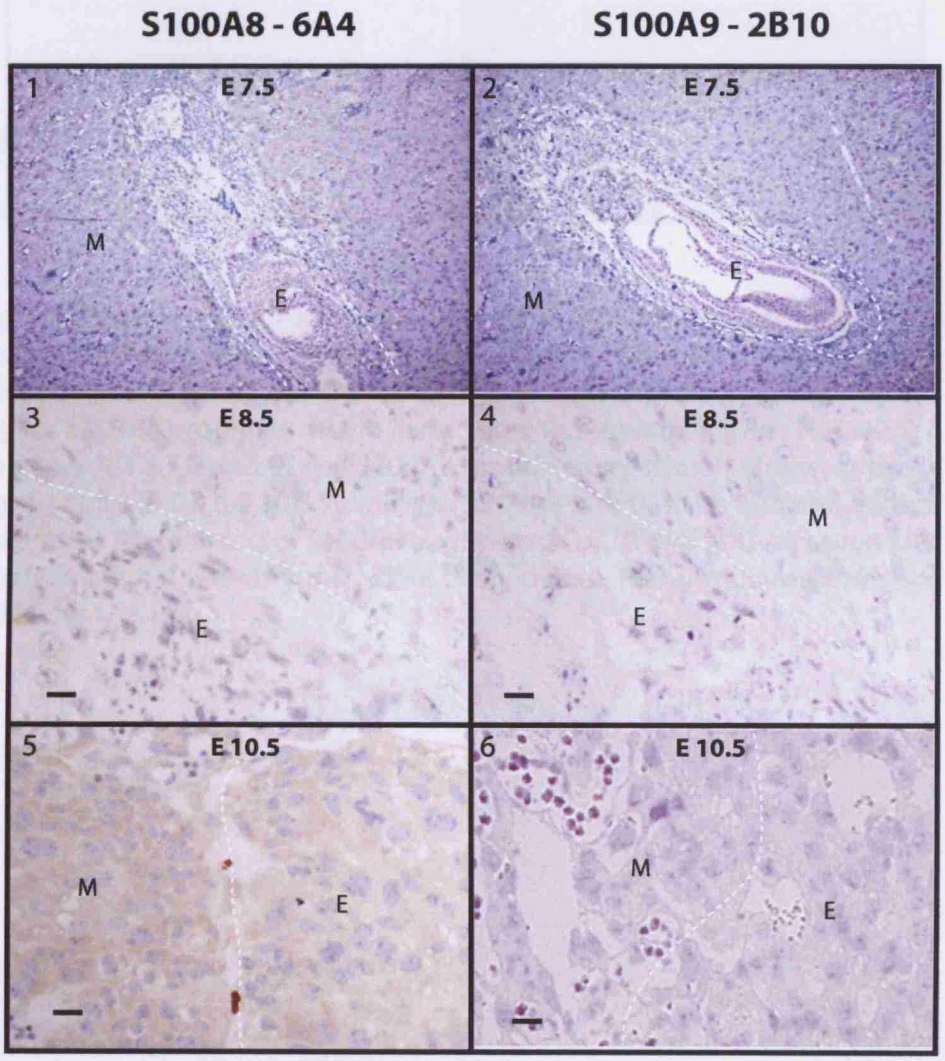
4.2.1 Decidual cells do not seem to stain positively for S100A8 protein

Expression of S100A8 mRNA in decidual tissue is well established in Passey et al 1999, Hobbs et al 2003 and this report. The data presented in this thesis show that the S100A8 mRNA expression is in the maternal decidual tissues. One issue that has not previously been addressed is whether the S100A8 mRNA expression is translated to protein expression. It is known that S100A8 mRNA is stable in S100A9 null myeloid cells but no S100A8 protein is present in these cells (Hobbs, May et al. 2003). This shows that there may be multiple levels of control for S100A8 protein expression. S100A8 may depend on a binding partner for stability as in the case of myeloid cells where S100A9 is required (Hobbs, May et al. 2003). In maternal decidual tissue it has been shown that there is a population of cells that express S100A8 mRNA but not S100A9 mRNA. The data suggest that if S100A8 protein is stably expressed in decidual tissues, it must be independent of its established heterodimeric partner, S100A9. The issue is critical in determining whether S100A8 has any role in postimplantation development. Only expression of stable S100A8 protein in embryonic tissues could explain the S100A8 null phenotype postulated in Passey et al 1999. It is also possible that S100A8 protein is expressed in maternal tissues and could show a role for S100A8 in decidual tissue biology. Another possibility is that S100A8 mRNA expression is triggered by events in the decidua and developing placenta but that this does not result in translated protein and therefore no function for S100A8. It is even possible that there is no constitutive role for S100A8 and that S100A8 protein expression is controlled by a stress induced pathway. To examine these possibilities we probed S100A8 and S100A9 protein expression in postimplantation decidual and embryonic tissues using immunohistochemistry as shown in fig 4.1.

A


S100A8 - 6A4
S100A9 - 2B10
1
Spleen
2
Spleen

B


S100A8 - 6A4
S100A9 - 2B10
1
E 7.5
M
E
2
E 7.5
E
M
3
E 8.5
M
E
4
E 8.5
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E
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E 10.5
M
E
6
E 10.5
M
E

B -continued

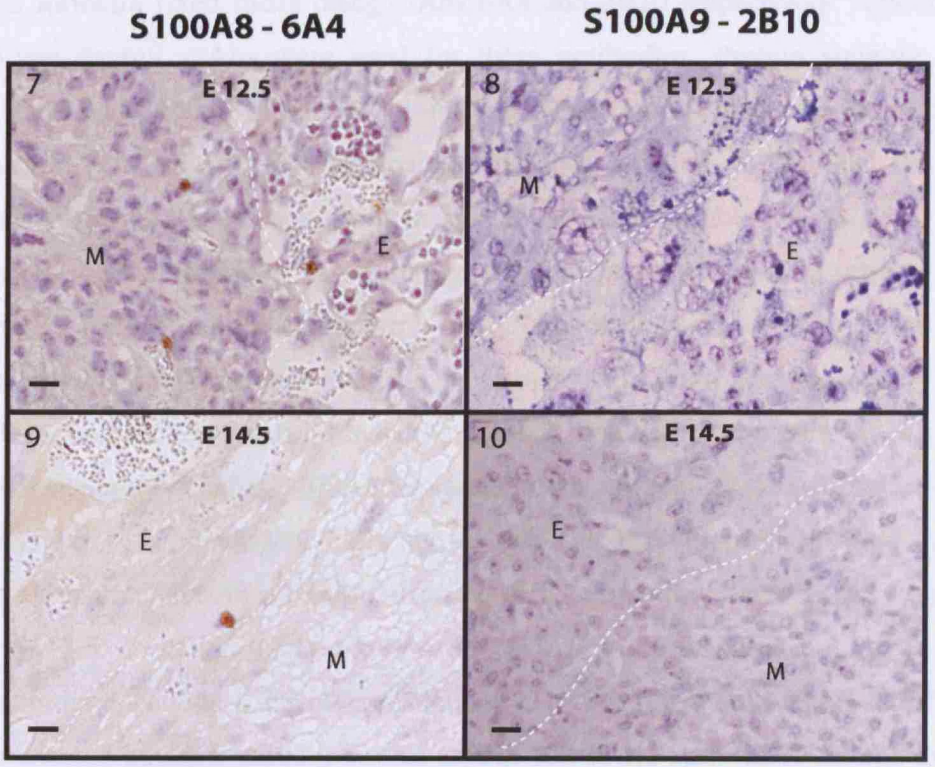


Fig 4.1 Immunohistochemistry of maternal decidual tissue. Immunohistochemistry of maternal decidual tissue. Positive control tissue spleen was stained for S100A8 (A1) and S100A9 (A2) protein expession. Immunostaining for S100A8 protein is shown in decidual tissues at E 7.5, 8.5, 10.5, 12.5 and 14.5 (B1,3,5,7 and 9) and S100A9 protein expression is shown at the same stages (B 2,4,6,8,10). Staining of S100A8 is with mAb 6A4 and anti-rat secondary Ab. Staining of S100A9 is with mAb 2B10 and anti-rat secondary Ab. M-maternal, E-embryonic, white dashed line - foetal-maternal interface. Scale bar = 20μM.

S100A8 and S100A9 protein expression was probed on E 8.5, 10.5 12.5 and 14.5 formalin fixed slides using mAbs 6A4 and 2B10 respectively. Relevant isotype control mAbs were used for these antibodies. Protein staining of S100A8 and S100A9 is confirmed in the positive control tissue spleen where myeloid cells are present and positive for both proteins. In decidual tissue S100A9 protein expression is restricted to a small number of cells at the maternal/foetal interface at all stages. This illustrates a similar pattern to S100A9 mRNA expression shown in fig 3.5 and is highly likely to represent a neutrophil population. An identical pattern of expression can be seen with S100A8 protein, which reinforces the myeloid cell expression pattern. In the wider maternal decidua S100A8 expression does not appear to occur within a particular cell population. This shows that the mesometrial decidual cell population of S100A8 mRNA expressing cells shown by *in situ* hybridisation in fig 3.5 does not stably express S100A8 protein. The general level of background staining is raised in decidua at E 10.5 and it was not known if this represented positive staining or was artefactual. It did seem clear though that S100A8 mRNA is not always correlated to S100A8 protein staining. These data show that at least at certain stages S100A8 mRNA is expressed but S100A8 protein is not evident. This would be consistent with expression of S100A8 mRNA in S100A9 null myeloid cells where no S100A8 protein is found despite S100A8 mRNA expression (Hobbs, May et al. 2003).

4.2.2 A transient expression of S100A8 protein can be seen between E 9.0 and E 11.5 in wildtype embryonic yolk sac.

Expression of S100A8 protein could not be clearly shown in maternal decidua at stages where high S100A8 mRNA expression can be shown. It was also of interest whether embryonic tissues express S100A8 protein, as expression in wildtype tissues would be key to determining the nature of any S100A8 null lethal phenotype. This was not previously been examined in the S100A8 null model. There has been no report of embryonic S100A8 protein expression prior to E 12.5, where it is seen in conjunction with S100A9 protein in myeloid cells of the embryonic liver. If S100A8 null embryos are lethal

postimplantation, it should be possible to see S100A8 protein expression in embryo-derived tissues at a stage close to the proposed lethality. The next two figures show the results of embryonic tissue immunohistochemistry. Staining in yolk sac is as shown in fig 4.2.

S100A8 and S100A9 protein expression was probed on E 8.5, 9.5 10.5 and 11.5 on formalin fixed slides using mAbs 6A4 and 2B10 respectively. Relevant isotype control mAbs were used for these antibodies. Positive control tissue spleen shows both S100A8 and S100A9 protein expression in myeloid cells in the red pulp regions. Staining of E 7.0 embryos showed no S100A8 expression as previously reported in Passey et al 1999 (not shown). Staining of embryonic tissue at E 8.5 – E 11.5 shows that there is a transient expression of S100A8 protein at E 9.5 and E 10.5 in yolk sac blood islands. At E 8.5 and E 11.5 there is no S100A8 protein expression in blood islands showing the transient nature of the expression. S100A9 protein expression cannot be seen in the yolk sac blood islands at any stage.

4.2.3 A transient expression of S100A8 protein can be seen in E 11.5 foetal liver.

Expression of S100A8 and S100A9 protein in foetal liver at E 12.5 is well established and due to nascent myeloid cells (Lagasse and Weissman 1992). Expression prior to this stage of either S100 protein has not been shown in any study to date. It is known that yolk sac cells can colonise the foetal liver starting at E 10.5. This colonisation facilitates liver development and transfers embryonic haematopoiesis to the foetal liver. Initially haematopoiesis in the liver is restricted to myeloid and erythrocyte lineages reflecting the status of cells migrating from the yolk sac. Monocytes and macrophage lineages can be seen in foetal liver as early as E 10.0 and accumulate throughout development (Morris, Graham et al. 1991). At E 11.5 HSCs migrate to the liver from, potentially, the yolk sac, the placenta and AGM region. It would be of interest whether S100A8 protein expression is seen prior to E 12.5 as yolk sac cells at

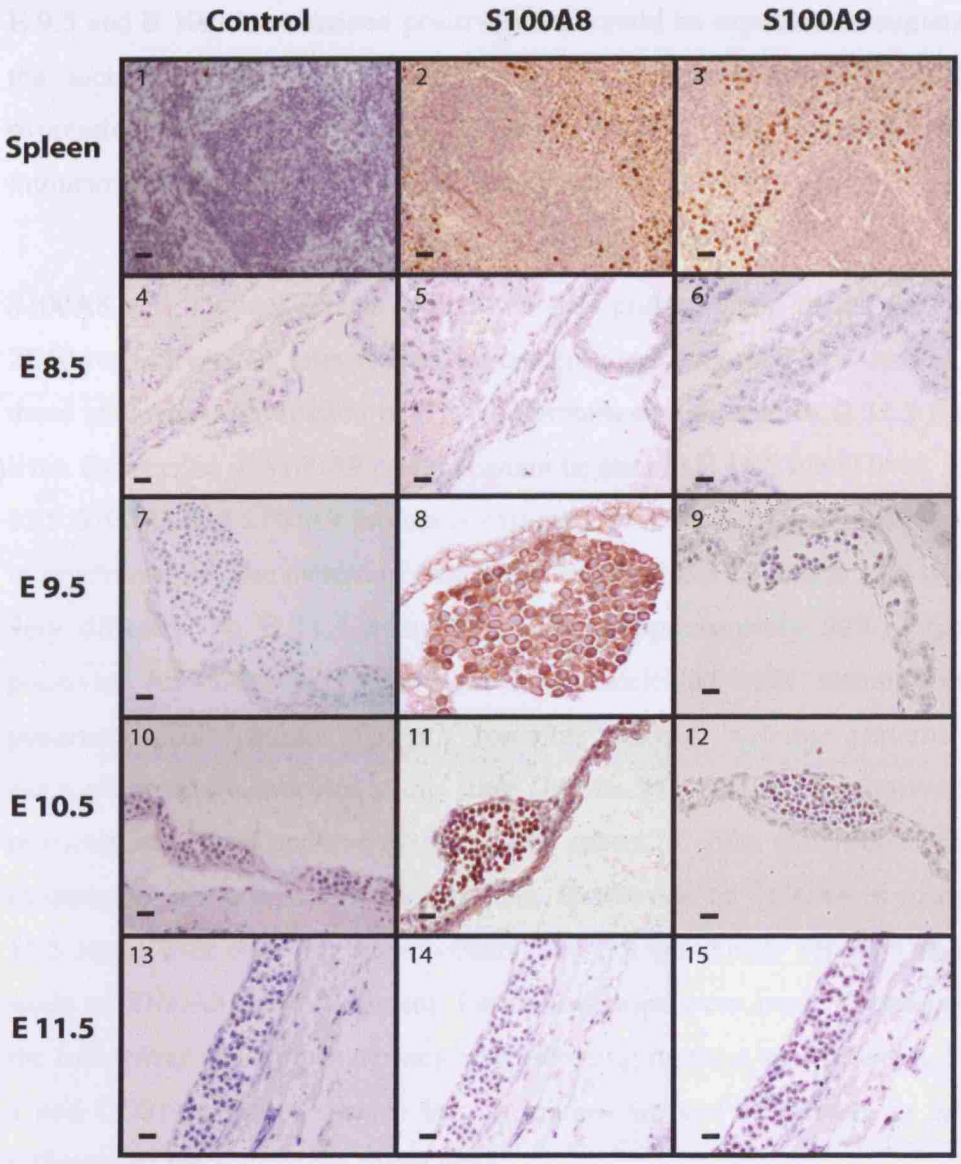


Fig 4.2 Expression of S100A8 protein in embryonic yolk sac. immunohistochemistry of spleen (1-3) and yolk sac samples at E 8.5 (4-6), E 9.5 (7-9), E 10.5 (10-12), and E 11.5 (13-15). Samples are stained with either control IgG (1,4,7,10 and 13) Ab 6A4 for S100A8 (2,5,8,11 and 14) or Ab 2B10 for S100A9 (3,6,9,12 and 15). Scale bar = 20μM.

E 9.5 and E 10.5 have stained positively and could be expected to migrate to the foetal liver prior to E 12.5. To assess this possibility, foetal liver expression of S100A8 and S100A9 protein was probed using immunohistochemistry at E 11.5 and 12.5 as shown in fig 4.3.

S100A8 and S100A9 protein expression was probed using mAbs 6A4 and 2B10 respectively. A relevant isotype control mAb was used as a control for these antibodies. Expression of S100A8 protein can be seen in E 11.5 foetal liver. Expression of S100A9 protein cannot be seen in E 11.5 foetal liver. At E 12.5 S100A8 and S100A9 protein is expressed in a well-documented manner in myeloid cells. The pattern of expression between E 11.5 and E 12.5 is also very different. At E 11.5 many liver cells (Approximately 50%) express positivity for S100A8 protein with large nucleated cells staining most positively. The pattern did not resemble known staining patterns of macrophage and monocytes at this stage (Morris 91). At E 12.5 expression is restricted to a small and evenly distributed subset of cells, which upon close examination are polymorphonuclear cells. Expression of S100A8 in many E 11.5 foetal liver cells is a novel observation not previously reported in any study of S100A8 in development. Further attempts were made to determine the foetal liver cell type expressing S100A8 using markers such as c-Kit, Sca-1 and CD31 (data not shown) but no marker worked effectively or could differentiate the cells of the foetal liver.

4.2.4 Flow cytometry shows no expression of S100A8 protein in E 9.5 and E 10.5 yolk sac

Immunohistochemistry showed a novel expression of S100A8 protein in yolk sac between E 9.5 and 10.5 and in foetal liver at E 11.5. This has not been seen in previous studies of S100A8 and S100A9 in early development. To determine whether a novel expression of S100A8 protein occurs in yolk sac cells intracellular flow cytometry was conducted on E 9.5 and E 10.5 yolk sac tissues. Flow cytometry, if successful, could make use of Abs for S100A8 protein in conjunction with cell marker Abs to determine which cell

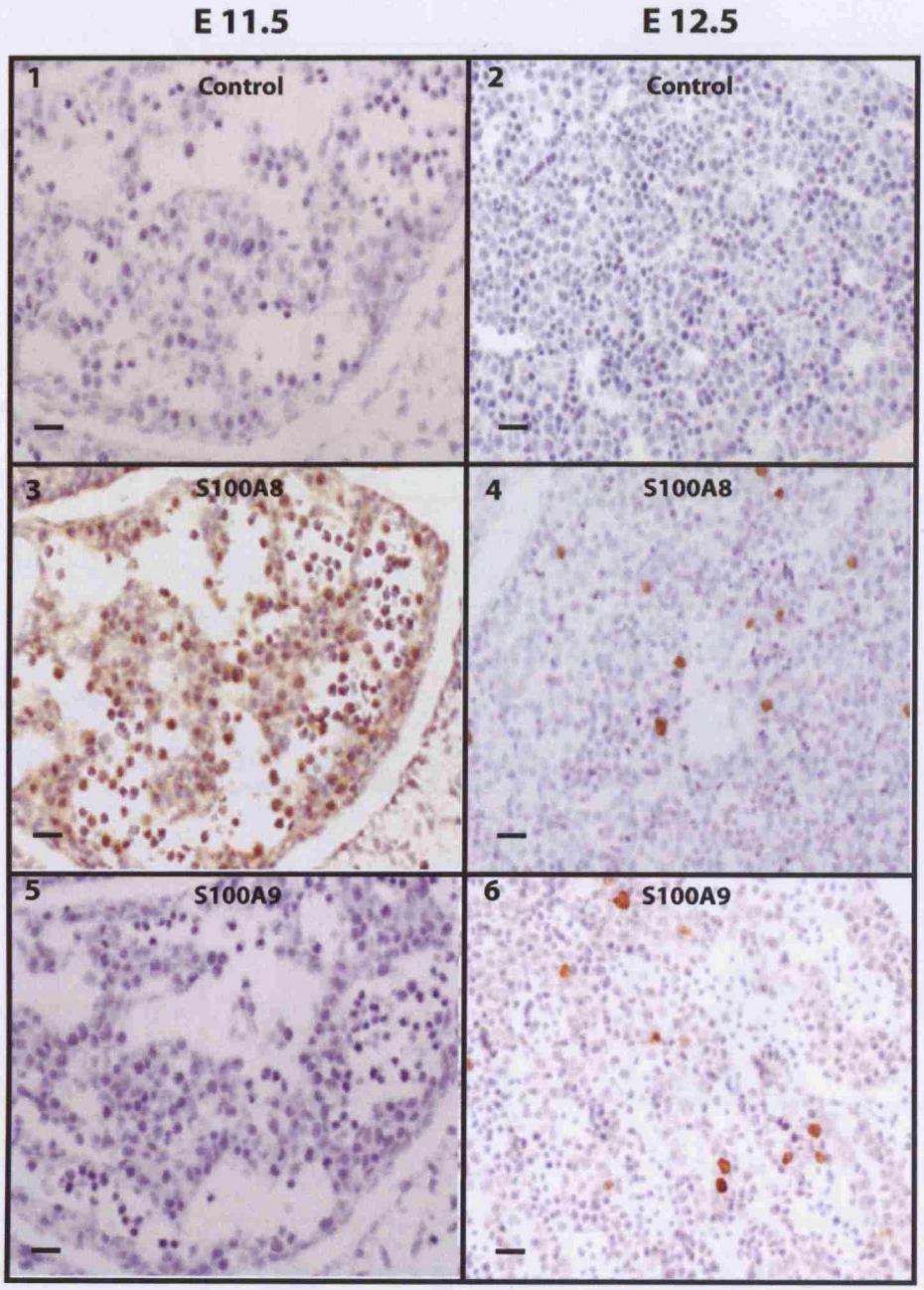


Fig 4.3 S100A8 and S100A9 protein expression in foetal liver. Immunohistochemistry of foetal liver at E 11.5 and E 12.5 treated with control IgG (A1,2) Ab 6A4-S100A8 (3,4) and Ab 2B10-S100A9 (5,6). All sections are treated with anti-rat secondary. Scale bar = 20μM.

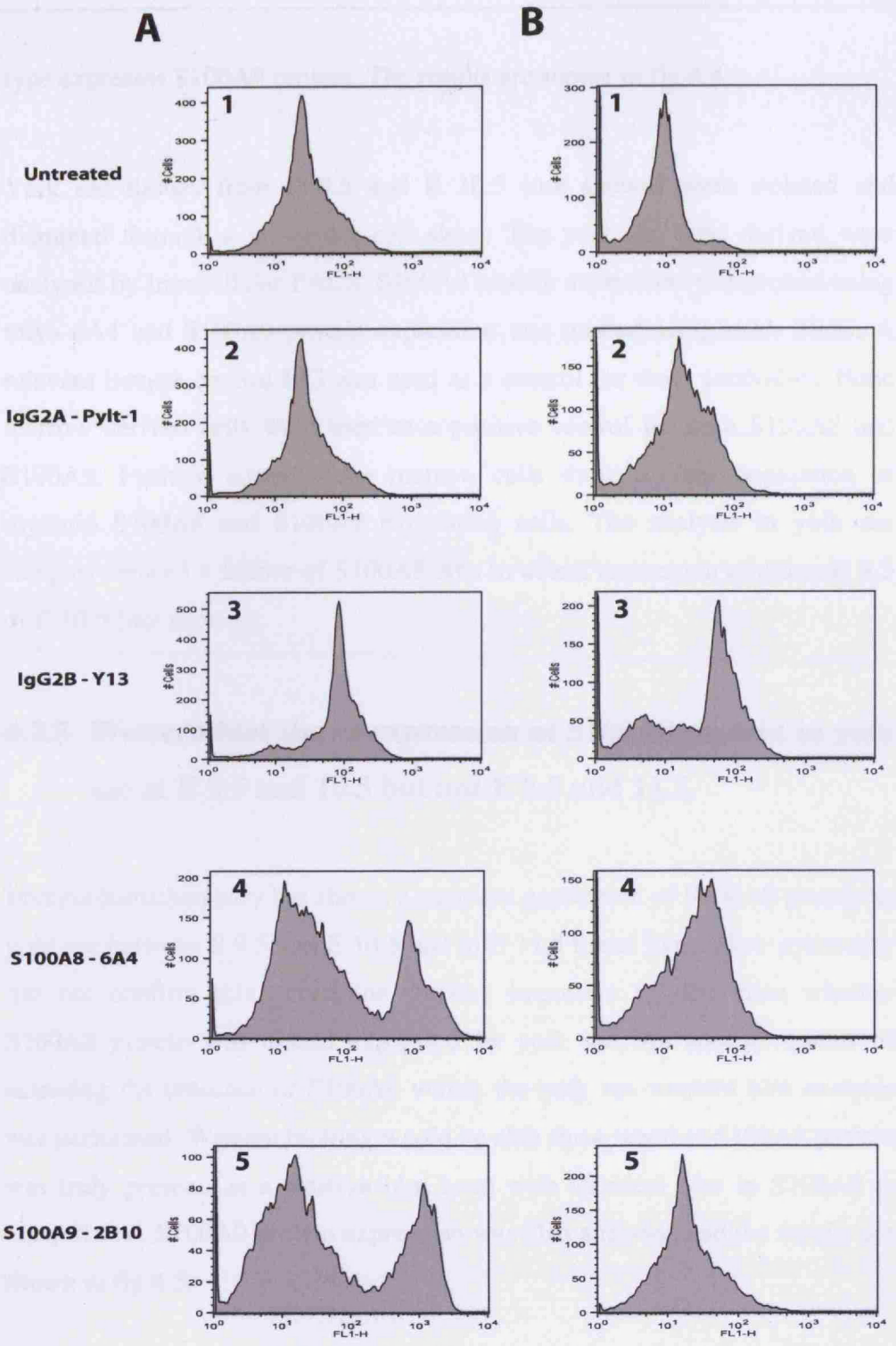


Fig 4.4 Flow cytometry of bone marrow and E 9.5 yolk sac samples. (A) Flow cytometry of A) bone marrow cells and (B) E 9.5 yolk sac cells treated with 1) No Ab 2) Pylt-1 3) Y13 4) 6A4 (S100A8) 5) 2B10 (S100A9). All cells are treated with appropriate secondary antibody.

type expresses S100A8 protein. The results are shown in fig 4.4.

Yolk sac tissues from E 9.5 and E 10.5 (not shown) were isolated and disrupted through a molecular cell sieve. The yolk sac cells derived were analysed by intracellular FACS. S100A8 protein expression was probed using mAb 6A4 and S100A9 protein expression was probed using mAb 2B10. A relevant isotype control IgG was used as a control for these antibodies. Bone marrow derived cells were used as a positive control for both S100A8 and S100A9. Positive control bone marrow cells show a clear population of myeloid S100A8 and S100A9 expressing cells. The analysis in yolk sac samples showed a failure of S100A8 Abs to detect expression at either E 9.5 or E 10.5 (not shown).

4.2.5 Western blot shows expression of S100A8 protein in yolk sac at E 9.5 and 10.5 but not E 8.5 and 11.5.

Immunohistochemistry has shown a transient expression of S100A8 protein in yolk sac between E 9.5 and E 10.5 and in E 11.5 foetal liver. Flow cytometry did not confirm this expression. It was important to determine whether S100A8 protein was indeed expressed by yolk sac. As another means of assessing the presence of S100A8 within the yolk sac western blot analysis was performed. Western blotting would be able show whether S100A8 protein was truly present, as a western blot band with identical Mw to S100A8 is unequivocal. S100A9 protein expression was also analysed and the results are shown in fig 4.5.

Wildtype yolk sac lysates from E 8.5, 9.5, 10.5 and 11.5 were analysed for S100A8 and S100A9 protein expression. Bone marrow lysate was used as a positive control for both S100A8 and S100A9 protein. S100A8 protein on western blots was probed with polyclonal antibody NH9. S100A9 is probed using mAb 2B10. Antibodies were controlled using relevant antibodies. Positive control bone marrow lysates stain positively for S100A8 and S100A9 protein. S100A8 expression was seen in E 9.5 and 10.5 samples and was

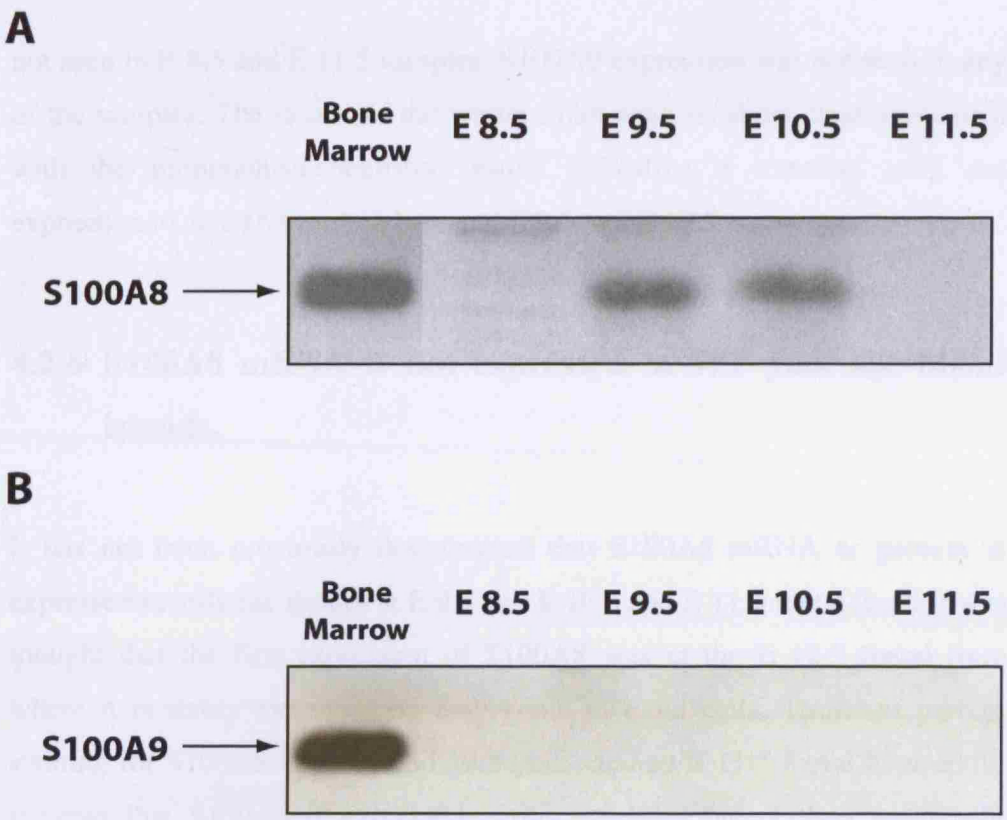


Fig 4.5 Western blot of yolk sac samples. Western blot showing S100A8 (A) and S100A9 B) protein expression in bone marrow lysates and yolk sac lysates at E 8.5, 9.5, 10.5 and 11.5. Staining of S100A8 is with Ab NH9 and anti-rabbit HRP conjugated secondary. Staining of S100A9 is with mAb 2B10 and anti-rat HRP conjugated secondary.

not seen in E 8.5 and E 11.5 samples. S100A9 expression was not seen in any of the samples. The results of the western blot analysis show clear agreement with the immunohistochemistry results indicating a transient yolk sac expression of S100A8 protein between E 9.5 and E 10.5.

4.2.6 S100A8 mRNA is not expressed in WT yolk sac blood islands.

It has not been previously documented that S100A8 mRNA or protein is expressed in yolk sac tissues at E 9.5 and E 10.5 and E 11.5 foetal liver. It was thought that the first expression of S100A8 was in the E 12.5 foetal liver where it is stably expressed by embryonic myeloid cells. Transient protein staining for S100A8 in E 9.5 and 10.5 yolk sac and E 11.5 foetal liver could indicate that S100A8 is expressed earlier in development than previously thought. This would also seem to correlate most closely with the report in Passey et al 1999 of S100A8 null lethality at E 8.5 and S100A8 null embryo loss by E 9.5. It would however show a very different cause of lethality than the one proposed in that report. Expression of S100A8 has been shown by immunohistochemistry and western blot but could not be confirmed by flow cytometry. Examination of S100A8 mRNA in foetal yolk sac should confirm expression of S100A8 protein in these tissues. S100A9 expression was also examined to further confirm whether S100A8 expression was indeed independent in the tissues examined as shown in fig 4.6.

S100A8 and S100A9 mRNA expression was probed using P_{32} radioactively labelled anti-sense probes for S100A8 and S100A9 mRNA. S100A8 and S100A9 mRNA were both detected in myeloid cells in E 12.5 foetal liver validating the probe. It can clearly be seen that no expression of S100A8 mRNA occurs in the yolk sac at any stage examined. No S100A8 mRNA expression could be seen in the foetal liver at E 11.5 (not shown). No S100A8 mRNA expression can be seen in any foetal tissues prior to the myeloid expression at E 12.5 in the foetal liver. This lack of expression can be compared to the high expression levels seen in the maternal decidua

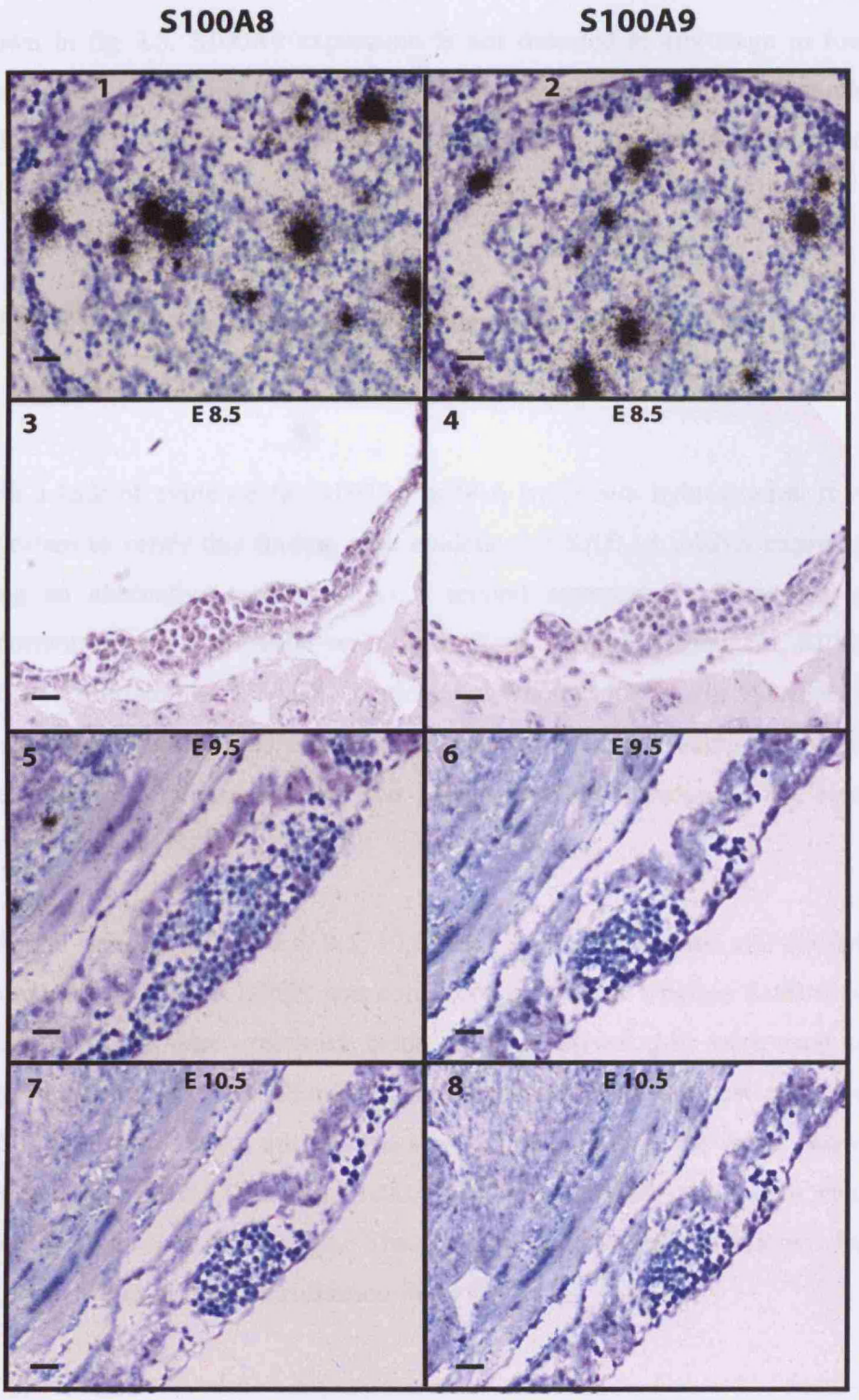


Fig 4.6 S100A8 and S100A9 mRNA expression in embryonic yolk sac. In situ hybridisation on foetal liver (1,2) and yolk sac tissues (3-8) with S100A8 (1,3,5,7) and S100A9 probes (2,4,6,8) at E 8.5 (3,4), E 9.5 (5,6) and E 10.5 (7,8). Scale bar = 20μM.

shown in fig 3.5. S100A9 expression is not detected at any stage in foetal tissues prior to myeloid cells in E 12.5 foetal liver. The evidence shows clear lack of any S100A8 or S100A9 mRNA expression in the foetal system prior to E 12.5 as has been previously reported.

4.2.7 RT-PCR of yolk sac samples show no S100A8 mRNA at E 8.5 – E 11.5

With a lack of evidence for S100A8 mRNA by *in situ* hybridisation it was important to verify this finding with evidence of S100A8 mRNA expression using an alternative technique. As a second approach to answering this important question RT-PCR was used. Examining S100A8 and S100A9 mRNA expression by RT-PCR should show whether the *in situ* hybridisation results were correct. This has the advantage of clearly looking at correctly sized specific bands for S100A8 and S100A9 mRNA expression. The results are as shown in fig 4.7

Yolk sac samples from E 8.5, 9.5, 10.5 and 11.5 were extracted and disrupted through a cell sieve. RT-PCR was conducted to analyse whether S100A8 and S100A9 mRNA were expressed. Bone marrow derived cells were used as a positive control for S100A8 mRNA. Bone marrow controls show expression of S100A8 and S100A9 mRNA. No stage of embryo yolk sac tissue showed expression of S100A8 mRNA. S100A9 mRNA expression was also absent from the yolk sac at these stages. These results confirm the observations from S100A8 mRNA *in situ* hybridisation in fig 4.4.

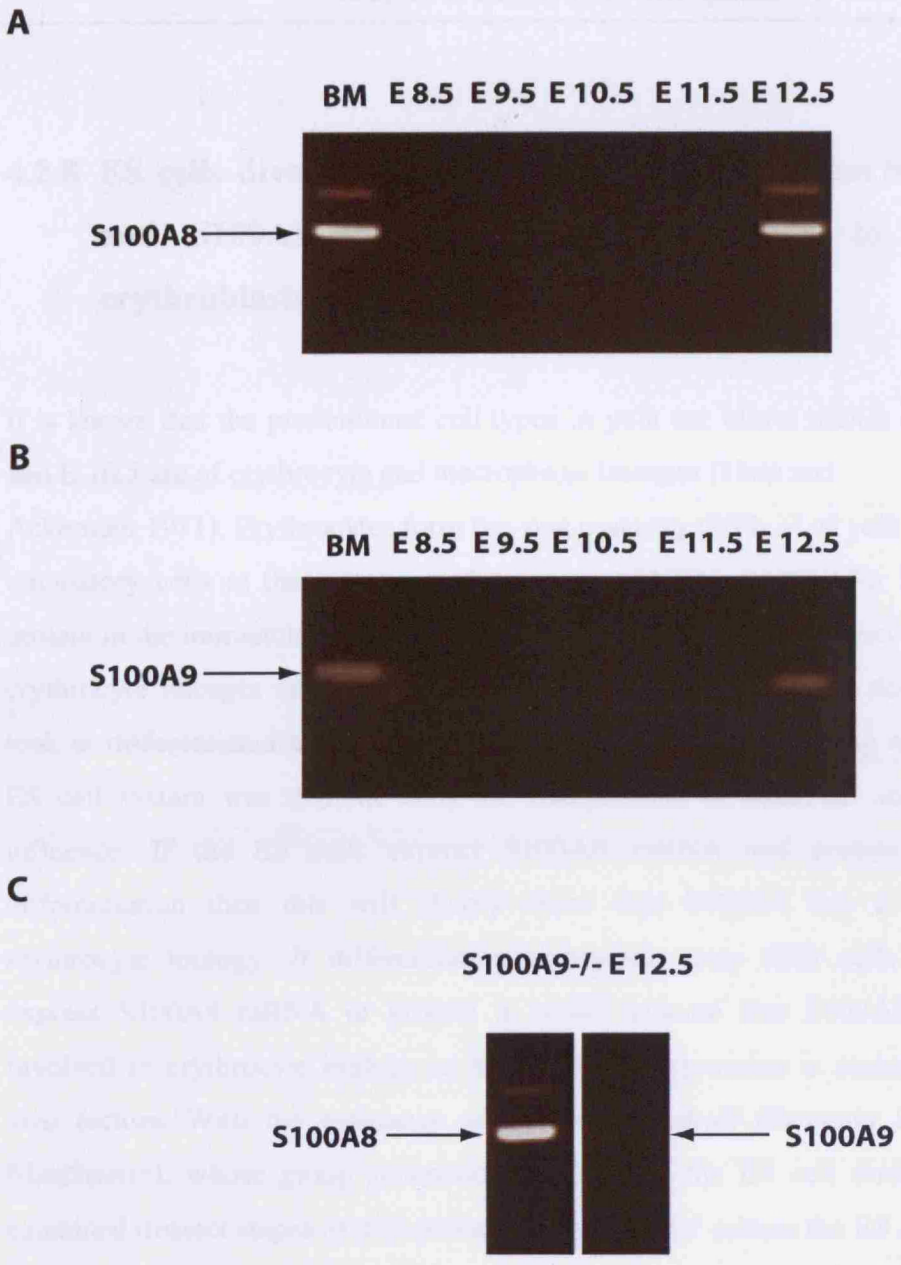


Fig 4.7 RT-PCR of yolk sac samples. S100A8 A) and S100A9 B) mRNA expression in yolk sac samples at E 8.5, 9.5, 10.5, 11.5 and E 12.5 embryo sample.. C) Expression of S100A8 and S100A9 mRNA in S100A9 null tissue sample at E 12.5.

4.2.8 ES cells diverted to erythroblast lineage express S100A8 and S100A9 mRNA after differentiation to "red" erythroblasts.

It is known that the predominant cell types in yolk sac blood islands at E 9.5 and E 10.5 are of erythrocyte and macrophage lineages (Haar and Ackerman 1971). Erythrocytes form the vast majority (95% +) of yolk sac and circulatory cells at these stages and so were certainly positive for S100A8 protein in the immunohistochemistry data shown in Fig 4.4. To assess whether erythrocyte lineages are S100A8 mRNA and protein positive we decided to look at differentiated erythrocyte lineages. The advantage of using a murine ES cell system was that the cells are independent of maternal and foetal influence. If the ES cells express S100A8 mRNA and protein during differentiation then this will clearly show that S100A8 has a role in erythrocyte biology. If differentiating embryonic stem (ES) cells do not express S100A8 mRNA or protein it could indicate that S100A8 is not involved in erythrocyte biology or that S100A8 expression is caused by *in vivo* factors. With the assistance of Valerie Kouskoff (Paterson Institute, Manchester), whose group generated and cultured the ES cell samples we examined distinct stages of differentiation. At day 0 of culture the ES cells are undifferentiated and must undergo a process, which takes them through processes highly similar to haematopoiesis *in vivo*. By day 2-3 some of the cells display characteristics of haemangioblast cells. By day 4 the ES cells have developed into erythroid precursors. By day 6 fully differentiated erythrocytes can be seen (Keller, Kennedy et al. 1993). The results of RT-PCR on the stages of differentiation are shown in fig 4.8.

ES cells in culture were diverted to the erythrocyte lineage as described in Kouskoff et al 2005. Cell samples were taken at day 0, 1, 4, 5 and 6 of culture. RT-PCR was conducted on the samples to analyse S100A8 and S100A9 mRNA expression. Bone marrow derived cells were used as a positive control for both S100A8 and S100A9 mRNA. No S100A8 or S100A9 mRNA

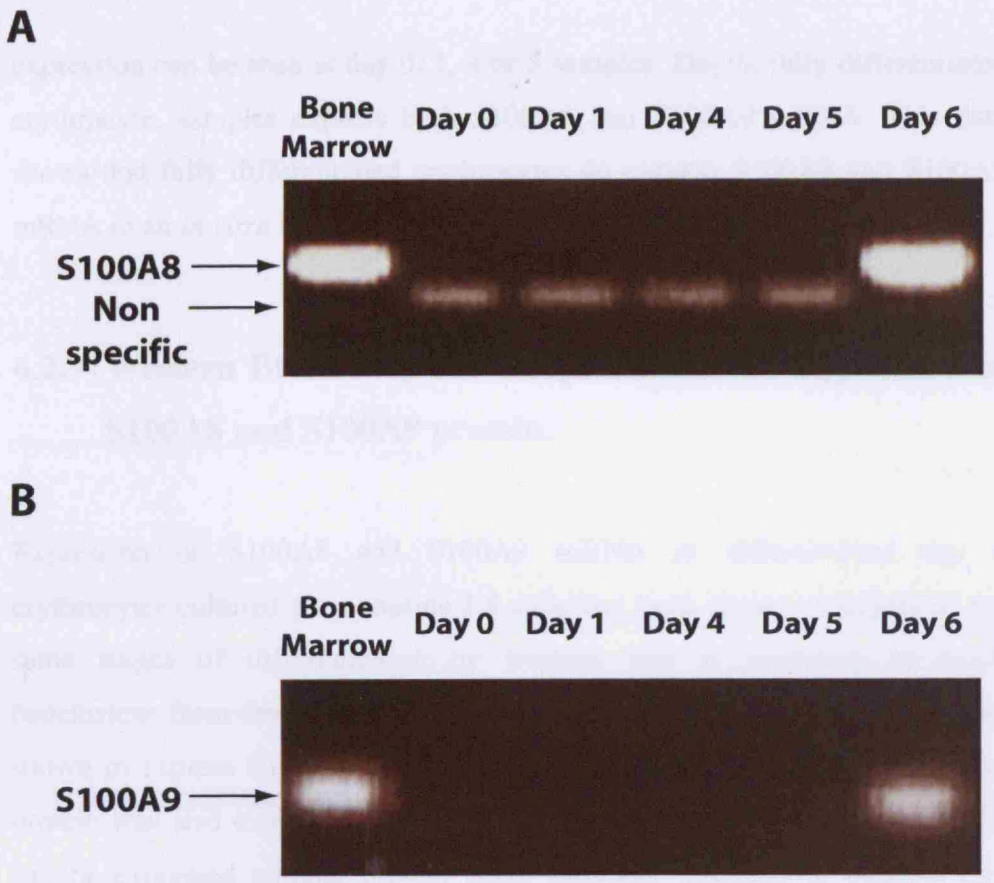


Fig 4.8 RT-PCR of stem cell derived cell samples. RT-PCR of control bone marrow and samples derived from embryonic stem cells at Day 0 and differentiated through erythrocytes at day 6. RT-PCR shows expression of S100A9 (A) and S100A9 (B).

expression can be seen at day 0, 1, 4 or 5 samples. Day 6, fully differentiated erythrocyte, samples express both S100A8 and S100A9 mRNA. This data shows that fully differentiated erythrocytes do express S100A8 and S100A9 mRNA in an *in vitro* culture system.

4.2.9 Western Blot of ES cell samples shows no expression of S100A8 and S100A9 protein.

Expression of S100A8 and S100A9 mRNA in differentiated day 6 erythrocytes cultured from murine ES cells has been shown. Analysis of the same stages of differentiation by western blot is necessary to reach conclusions from the culture experiments. In particular the day 6 sample was shown to express S100A8 and S100A9 mRNA so it was if interest whether protein was also expressed. As we have seen in earlier data S100A8 mRNA can be expressed without protein being detected. Analysis of S100A8 and S100A9 protein expression will show whether these proteins are stably expressed in this *in vitro* erythrocyte system. The samples used in this experiment were also derived from our collaboration with the group of Valerie Kouskoff. The results are shown in fig 4.9.

Cultured ES cell samples at 0,1,4,5 and 6 days were disrupted in lysis buffer and analysed by western blot for S100A8 and S100A9 protein expression. Bone marrow lysate is used as a positive control for both S100A8 and S100A9 protein. S100A8 on western blot is probed with rabbit polyclonal antibody NH9. S100A9 is probed using mAb 2B10. Antibodies were controlled using a relevant secondary antibody. Positive control bone marrow cells stained positive for both S100A8 and S100A9 protein. No expression of S100A8 or S100A9 expression was seen at any stage of ES cell differentiation including day 6 differentiated erythrocytes. These data show that S100A8 and S100A9 mRNA is not stably translated to protein expression in ES cell derived, differentiated erythrocytes.

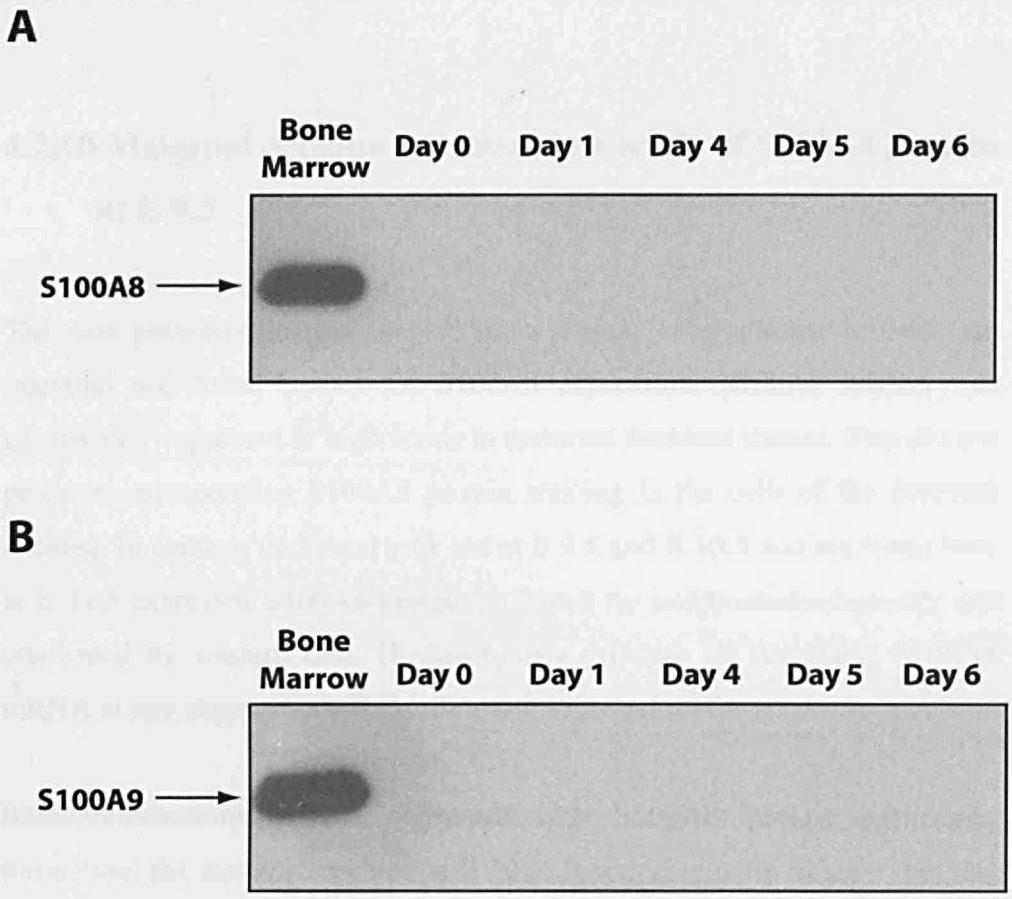


Fig 4.9 Western Blot of bone marrow and stem cell samples. Staining of S100A8 A) and S100A9 B) protein expression in bone marrow positive control and Day 0,1,4, 5 and 6 of culture. Staining of S100A8 is with Ab NH9 and anti-rabbit HRP conjugated secondary. Staining of S100A9 is with Ab 2B10 and anti-rat HRP conjugated secondary.

4.2.10 Maternal decidua expresses low levels of S100A8 protein at E 9.5

The data presented in this chapter show a clear contradiction between the maternal and foetal tissues for S100A8 expression. S100A8 mRNA was consistently expressed at high levels in maternal decidual tissues. This did not produce corresponding S100A8 protein staining in the cells of the maternal decidua. In contrast the foetal yolk sac at E 9.5 and E 10.5 and the foetal liver at E 11.5 expressed S100A8 protein as shown by immunohistochemistry and confirmed by western blot. However, they produce no detectable S100A8 mRNA at any stage observed.

Immunohistochemistry had suggested low S100A8 protein expression throughout the maternal decidua at E 10.5. It was originally thought that this could be raised background or artefactual, but was it possible that this represented widespread but very low expression of S100A8 protein. It was decided to look at the possibility of low level S100A8 protein expression at E 9.5 and 10.5 in the maternal decidua. It would be possible to explain the observations if maternal decidual tissue produced S100A8 protein, which was secreted and crossed through the decidua to the foetal circulation. Many reports on the functions of S100A8 have indicated an extracellular role (Passey, Xu et al. 1999) and some reports have suggested a transcellular role (Kannan 2003). To assess this possibility western blot analysis of maternal decidual tissues was conducted. The decidual tissues were obtained from the S100A9 null mouse, as the maternal decidua will contain small populations of myeloid cells that are positive for S100A8 and S100A9 protein in wildtype. Myeloid cells would inevitably contaminate any examination of S100A8 and S100A9 protein expression in maternal decidua. However, S100A9 null mice contained myeloid cells that lack S100A8 or S100A9 making it an elegant model to look at maternal decidual S100A8 protein expression without fear of contamination. E 11.5 decidua was included as a stage where no S100A8 is seen in the yolk sac blood islands. The results are shown in fig 4.10.

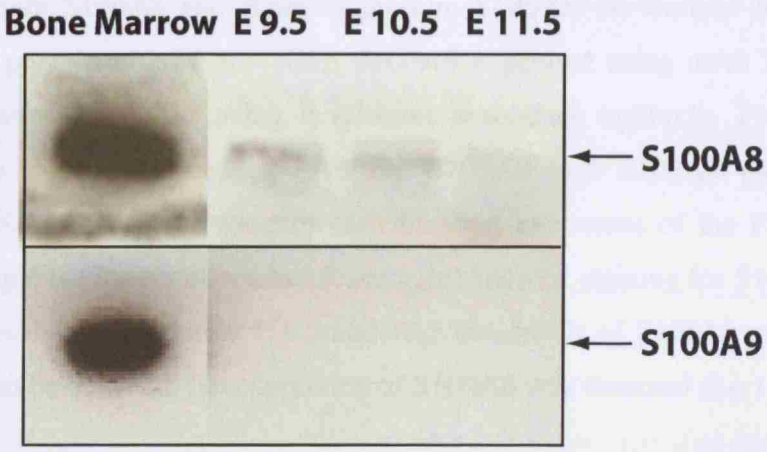


Fig 4.2.10 Western blot of maternal decidual tissue. Western Blot showing NH9 staining of S100A8 protein expression in E 9.5 10.5 and 11.5 maternal decidual samples. S100A9 staining shown with mAb 2B10.

S100A9 null maternal decidua at E 9.5 and E 10.5 was isolated and lysed for analysis by western blot. Wildtype bone marrow lysate is used as a positive control for both S100A8 and S100A9 protein. S100A8 on western blot is probed with polyclonal antibody NH9. S100A9 is probed using mAb 2B10. Antibodies were controlled using a relevant secondary antibody. Positive control bone marrow stained positive for S100A8 and S100A9 protein. Staining of S100A8 protein was not seen in short exposures of the E 10.5 decidual sample but longer exposures (overnight) showed staining for S100A8 protein. Thess data show that at E 9.5 and 10.5 low levels of S100A8 protein expression can be detected. No expression of S100A8 was detected at e 11.5.

4.2.11 S100A8 expressing cells in maternal decidua are not F4/80 positive macrophages

One possible cell type responsible for the S100A8 mRNA and protein expressing cells in the maternal decidua is macrophages. S100A8 expression can be seen in macrophages under inflammatory conditions (Hu, Harrison et al. 1996). This makes macrophages an interesting candidate cell type for the maternal decidual S100A8 mRNA and protein staining. Studies in chapter 3 of this thesis showing 7/4 staining in the decidua have indicated that other known S100A8-expressing immune cells such as monocytes and neutrophils are not responsible for the S100A8 expression in maternal decidua so it is important to rule out further cell types.

This analysis is balanced with the knowledge that macrophages are excluded from sites of implantation and development (Brandon 1993). It has been shown that macrophages are detrimental to the delicate balance of engraftment and rejection of the developing foetus by the maternal decidua. It was decided to reassess macrophage distribution in our S100A8 model to determine whether they are indeed excluded from the developing embryos or whether we may be looking at a macrophage expression of S100A8. This will also give reassurance that the S100A8 mRNA and protein expression we are seeing in the maternal decidua and haematopoietic tissues is not a

response to decidual inflammation or abnormal embryo growth. Macrophage distribution in the maternal decidua was probed by immunohistochemistry with pan macrophage marker F4/80 (Austyn and Gordon 1981) as shown in fig 4.11.

F4/80 protein expression was probed at E 7.5 8.5, 10.5 and 12.5 using mAb F4/80 (Serotech). A relevant mAb was used as a control for this antibody. Positive control tissue, E 12.5 foetal liver shows F4/80 expression in myeloid cells. Macrophages are not present in the maternal decidua at any stage examined. As documented they are excluded from the maternal decidual tissue postimplantation and cannot be the S100A8 mRNA and protein expressing cell type seen in maternal decidua.

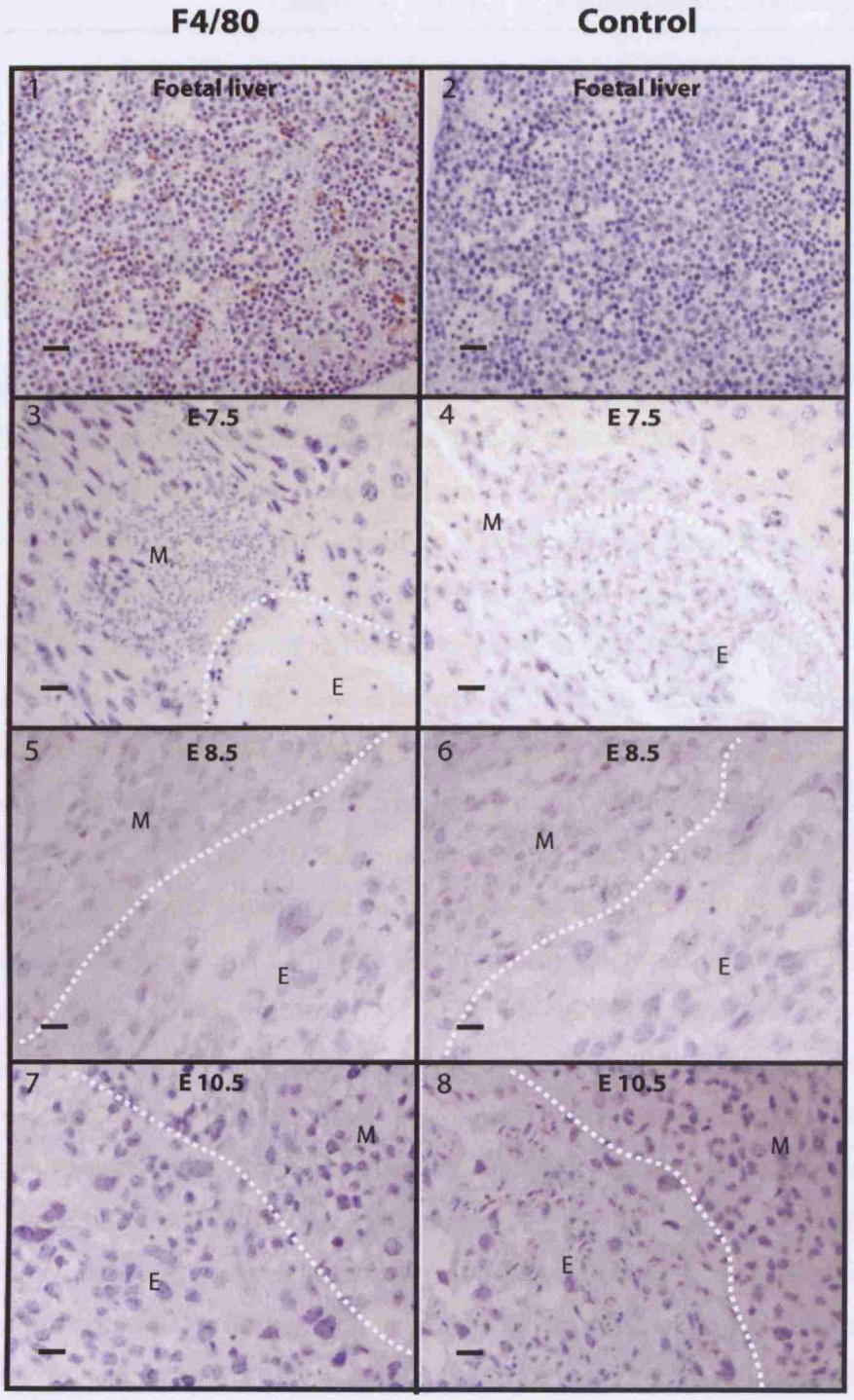


Fig 4.11 F4/80 Immunohistochemistry in maternal decidua. Macrophage marker F4/80 staining in positive control E 12.5 embryonic liver (1) and maternal decidual tissue at E 7.5 (3) E 8.5 (5) E 10.5 (7). Control tissues are also shown (2,4,6,8). F4/80 staining using F4/80 mAb followed by anti-rabbit secondary Ab. M-maternal tissue, E- embryo tissue. Scale bar = 20μM.

4.3 Discussion

4.3.1 Postimplantation role of S100A8

This chapter presents evidence of a complex situation for S100A8 expression in the maternal decidua and embryonic tissues postimplantation. The data in chapter 3 presented a previously unknown population of S100A8 mRNA expressing cells in the maternal decidua. The data in this chapter showed that the high S100A8 mRNA in the maternal decidua was not translated to comparable high levels of S100A8 protein in this tissue. It has been demonstrated though that low amounts of S100A8 protein were stably produced in the maternal decidua at E 9.5 and E 10.5. In contrast S100A8 protein is found in E 9.5 and E 10.5 embryonic yolk sac blood island cells and E 11.5 foetal liver. The S100A8 protein in these cells was demonstrated by immunohistochemistry and western blot analysis but not by flow cytometry. These cells do not express S100A8 mRNA at any observed point, which was shown by *in situ* hybridisation or RT-PCR. ES cells differentiating down the erythroid lineage did not express S100A8 or S100A9 mRNA or protein. Differentiated erythrocytes from ES cells, which should represent the predominant cell type of foetal circulation and yolk sac blood islands, did not express S100A8 and S100A9 protein although they did express S100A8 and S100A9 mRNA at the end stage of maturation. Finally it was shown that macrophages were probably not the S100A8 expressing population in the maternal decidua, as they are not detected in the area of S100A8 expression during the critical period.

4.3.2 Stability of S100A8 protein in the absence of S100A9

S100A9 null myeloid cells express S100A8 mRNA but do not stably express S100A8 protein (Hobbs, May et al. 2003). This observation suggests that S100A8 protein stability in myeloid cells is dependent on S100A9 expression (Hunter and Chazin 1998). It also suggests that S100A8 protein

homodimers do not occur in myeloid cells, as the conditions to produce such homodimers exist in S100A9 null mice. It has been proposed that in mice S100A8 does not have the necessary complementary dimerisation plane residues to form stable homodimers (Hunter and Chazin 1998). In development it has been shown, by studies including this one, that S100A8 mRNA is expressed in the absence of S100A9 mRNA expression in maternal decidua (Passey, Williams et al. 1999; Hobbs, May et al. 2003). This study confirms that observation and showed further that S100A8 protein was stable in the absence of S100A9. This finding raises the important question of how S100A8 protein was stabilised in the absence of S100A9. It shows that the state of S100A8 in myeloid cells and in embryo development is widely different. Homodimerisation of S100A8 is still a possibility although this is not energetically favourable (Hunter and Chazin 1998). It has been proposed that oxidation of S100A8 stabilises homodimers (Harrison, Raftery et al. 1999) and that this could occur in embryonic development (Passey, Williams et al. 1999). Another possibility is that there is an alternative binding partner for S100A8, in the absence of S100A9. A putative binding partner could shed light on the role of S100A8 in development and would be of great interest. Preliminary experiments to investigate a putative partner were inconclusive. Non-reducing western blots could show candidate bands, which might represent S100A8 homodimers or a heterodimeric complex. Preliminary experiments were undertaken to determine the nature of S100A8 in development but the results were not conclusive (data not shown). It is reasonable to conclude that S100A8 must form either homodimers or a unique complex with a previously unknown partner as no evidence exists for the stability of S100A8 monomers.

The stability of S100A8 highlights another issue raised by the data presented. Different techniques gave different answers when testing for the expression of S100A8 protein in yolk sac cells. Immunohistochemistry and western blot showed expression of S100A8 protein in the yolk sac at E 9.5 and E 10.5, but flow cytometry of the same cells did not show S100A8 protein expression. It is interesting that both western blot and immunohistochemistry present a picture of the cells fixed in place by either lysis buffer or by fixative,

whereas flow cytometry dilutes the cells into buffers before the fixative step. This may be the crucial factor in the conflicting results of these techniques with S100A8 protein either unstable or loosely associated with the yolk sac cells and hence lost in flow cytometry. Another possibility is that S100A8 was present in the fluid surrounding the cells and thus would have been present in western blot samples and was fixed with cells in immunohistochemistry but was lost in flow cytometry. It is also possible that the availability of S100A8 epitopes varies between the techniques. It is unclear how S100A8 in development is stabilised or whether it has a binding partner. It is therefore not clear whether the epitopes recognised by the S100A8 Abs are available in the techniques used to study expression. This problem is compounded by the necessary use of different Abs in different techniques due to the fact that no one Ab worked in all the techniques used.

4.3.3 S100A8 protein in foetal and maternal decidual tissues

Expression of S100A8 protein in yolk sac at E9.5 and E10.5 and foetal liver at E 11.5 was initially highly surprising and difficult to reconcile with the lack of S100A8 mRNA in those locations at any stage studied. In addition to this the lack of S100A8 mRNA, a lack of protein production in the ES cell derived erythrocytes also confirms that S100A8 protein is not expressed in this cell type. The expression of S100A8 and S100A9 mRNA in ES cells at day 6 of differentiation could be explained by the presence of nascent myeloid cells within the culture. The culture conditions favour erythrocyte differentiation but small myeloid populations could be present (Kouskoff, Lacaud et al. 2005). There remains a slight possibility that S100A8 mRNA expression is transient and S100A8 protein was produced by yolk sac and foetal liver cells and that this was never observed. It was far more likely that yolk sac and foetal liver cells do not produce the S100A8 protein and the source lies elsewhere. If the source does lie elsewhere it is of interest how the S100A8 protein ends up in the yolk sac and foetal liver cells.

It was also surprising that despite high S100A8 mRNA expression in maternal

decidual tissue, that there was not concomitant S100A8 protein expression at all stages. This indicates at least 2 levels of control for S100A8 protein expression with mRNA induced at E 7.5 before protein is shown at E 9.5. However, expression of S100A8 protein in maternal decidual tissue was eventually shown to occur at low levels by western blot, which confirmed the impression that S100A8 mRNA expression is translated. The stage specific expression of S100A8 protein in decidual tissue suggests a mechanism for regulating the translation of S100A8 mRNA as it is expressed throughout development yet S100A8 protein is not found at most stages. Co-ordination of signals for transcription and translation appear to be required. The exact nature of the signalling events that control S100A8 mRNA and protein expression could give insight to the function of S100A8 and it would be desirable to investigate these control mechanisms.

Our data show a possible novel role for S100A8 in development at E 9.5 to E 11.5. Combining the data from chapter 3, where we see extensive maternal decidual S100A8 mRNA expression throughout development, with the transitory expression of S100A8 protein in embryonic tissues between E 9.5 and E 11.5 a novel mechanism of S100A8 action can be proposed. Expression of S100A8 protein in the maternal decidual tissues seems certain and its presence in the foetal yolk sac and liver strongly shows the possibility of S100A8 export across the placenta into foetal tissues as illustrated in fig 4.12. With no explanation for the embryonic expression of S100A8 and a known source of S100A8 mRNA and protein nearby in the maternal decidua it is a strong possibility. An interesting hypothesis is that S100A8 protein could be exported across the placenta at E 9.5 and E 10.5 where it is picked up by foetal erythrocytes in the yolk sac as shown in fig 4.1. This does not occur prior to E 9.5 as the maternal decidua expresses only mRNA and not protein and it does not occur after E 11.5 as no protein can be detected in yolk sac. Yolk sac erythrocytes are known to migrate to the foetal liver at E 10.5 and S100A8 expression is seen at E 11.5 in the foetal liver. The exact correlation of S100A8 protein expression in the maternal decidua and embryonic yolk sac is too precise in timing to be easily dismissed as coincidence.

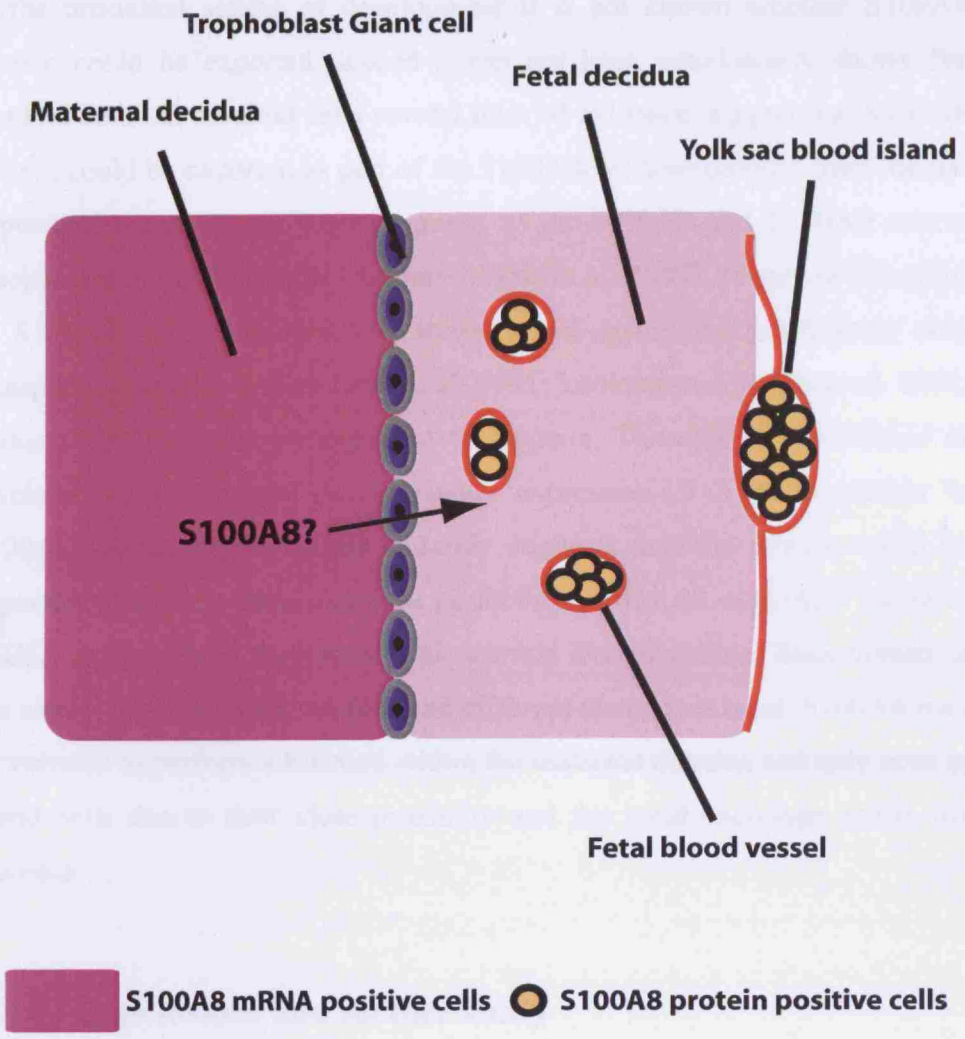


Fig 4.12 Separation of S100A8 message and protein expression at E 9.5 and E 10.5. Representation of the divergence of S100A8 message and protein expression. Maternal decidual cells (purple) express high S100A8 mRNA levels whereas S100A8 protein positive cells in the yolk sac and circulation. The model proposes a possible link between the two phenomenon.

In the unstudied setting of development it is not known whether S100A8 protein could be exported, indeed it has not been conclusively shown for myeloid cells. In myeloid cells several lines of evidence suggest that S100A8 protein could be exported as part of the S100A8/A9 heterodimer. Microtubule dependent secretion has been proposed as an S100A8 and S100A9 export mechanism in myeloid cells (Rammes, Roth et al. 1997). Proposed functions of S100A8 as chemo-attractant, antimicrobial agent and arachidonic acid transporter (Sohnle, Collins-Lech et al. 1991; Lackmann, Cornish et al. 1992; Kannan 2003) all rely on export of the protein. The combined evidence of myeloid cell export and developmental expression of S100A8 protein in S100A8 mRNA negative cells certainly suggests that the protein could be exported. The only other plausible possibility is that an extremely transient mRNA expression in embryonic yolk sac and liver may have been missed in our studies despite repeat samples and different techniques used. S100A8 may be secreted to perform a function within the maternal decidua and only seen in foetal cells due to their close proximity and the rapid exchange across the placenta.

4.3.4 Conclusions and further study

Evidence has been presented to support a model of S100A8 protein expression in the maternal decidua at E 9.5 and E 10.5, one stage among a continuous S100A8 mRNA expression from E 7.5 to E 14.5. Evidence has been presented that S100A8 protein is found at E 9.5 and E 10.5 in the yolk sac and at E 11.5 in the foetal liver. No evidence was found to support S100A8 mRNA expression in these tissues. ES cell derived cell equivalents do not express S100A8 protein. The model proposed to link these events is export of S100A8 protein from the maternal decidua to cells within the yolk sac and placenta before those cells migrate to the foetal liver. No evidence presented in this chapter supports the idea of an S100A8 null lethality at E 9.0. Studies of preimplantation development may be key to discovering the S100A8 null lethal phenotype

CHAPTER 5

5 Preimplantation studies of S100A8

5.1 Introduction

Chapter 4 of this thesis examined postimplantation development and found no results supporting a critical role for S100A8, and instead showed evidence of a maternal role for the protein in the developing placenta. Data from these studies along with evidence from chapter 3 showing significant loss of S100A8 null embryos indicated a preimplantation lethal phenotype for the S100A8 null mouse prior to E 4.5. In order to determine the exact phenotype of the S100A8 null mouse model preimplantation studies were needed. This has been a previously unstudied area for S100A8 and there is no indication as to what the role of the protein might be in this context. The purpose of the work described in this chapter has been to attempt definition of the exact timing of the S100A8 null lethal phenotype and to examine the cause of lethality.

Oocyte maturation occurs both within the ovary and in the uterine horn. The first round of meiosis occurs within the ovary to transform primary oocytes with germinal vesicles into secondary oocytes. Further meiosis produces a fully mature oocyte ready for fertilisation. After fertilisation, cell division progresses from 1-cell to 2-cell and on to the 4-cell stage. At the 8-cell stage a process called morulation occurs where cell-cell contacts tighten and differentiation begins. Morulation is the first morphogenic step in differentiation from totipotent blastomeres to the first differentiated cell types, the trophoblast and inner cell mass (ICM) (Johnson and Ziomek 1981). Morulae then undergo further morphogenic changes to become

blastocysts. A liquid filled cavity called the blastocoele develops in the centre of the morula (Barcroft, Offenberg et al. 2003). The trophoblast cells become a flattened monocellular layer surrounding the blastocoele with the inner cell mass polarised against a section of trophectoderm. Successful blastulation prepares an embryo to hatch from the zona pellucida and undergo implantation.

In vitro culturing of preimplantation embryos first became possible after work in the 1950s (Whitten 1956) showed that specific buffer conditions were required to support growth. Further advances in culture conditions now mean that it is possible to culture embryos from 1 cell through to hatched blastocysts ready to implant. *In vitro* culture does have an effect on embryos and can cause changes in global gene expression (Khosla, Dean et al. 2001) that differ from *in vivo* development. This would be expected, as it is not yet possible to fully recreate the conditions of the uterus in culture.

One of the aspects that must be borne in mind when investigating preimplantation development is the specialised genetic nature of gametes. Containing only one copy of the S100A8 gene at fertilisation, sperm and oocytes are essentially all either S100A8 wildtype or S100A8 null. This is an important point as one possible cause of preimplantation lethality is a non-redundant S100A8 gamete function. In maintaining our breeding line of S100A8 heterozygous mice, we have backcrossed S100A8 heterozygous mice with wildtype mice. Any S100A8 heterozygous offspring from backcrossing must have resulted from fusion of a wildtype gamete with an S100A8 null gamete. As we have used both male and female S100A8 heterozygous mice in backcrossing, it appears that S100A8 null sperm and oocytes are viable when paired with a wildtype gamete. It is evident that the combination of two S100A8 null gametes is necessary to cause lethality. Another aspect, which may influence the S100A8 null phenotype is the timing of the maternal zygotic transition, which in mice occurs at the 2-cell stage. This transition marks the first expression of the combined zygotic genome and the degradation of maternal oocyte transcripts and most maternal proteins. Prior to this stage all protein and transcripts are oocyte derived. The combination

of two S100A8 null gametes has proven necessary for S100A8 lethality and this would be expected to first occur at the 2-cell stage of development. While we acknowledge this information, it was also important not to prejudice our studies of the S100A8 null lethality as unexpected results could require flexible analysis.

5.2 Results

5.2.1 S100A8 protein is not expressed in testes.

Expression of S100A8 has not been previously studied in gamete differentiation processes. It is possible to state that logically there should not be a problem in gamete production or function as outlined in 5.1. In order to further eliminate the possibility of a role for S100A8 in gamete cells it was decided to probe testes for S100A8 protein expression by immunohistochemistry. Testes were chosen as they present the full stages of differentiation in spermatogenesis and it was possible to examine every step with one experiment. The results are shown in fig 5.1.

S100A8 and S100A9 protein expression was probed on testes in formalin fixed slides using mAbs 6A4 and 2B10 respectively. Relevant isotype control mAbs were used as controls for these antibodies. Protein staining of S100A8 and S100A9 is confirmed in the positive control tissue, spleen, where myeloid cells are present and positive for both S100 proteins. No expression of S100A8 or S100A9 was seen in any stage of spermatogenesis in testes. The data presented showed no reason to find fault with the logic that S100A8 null gametes are functional to the point of sperm differentiation in testes.

5.2.2 S100A8 null lethality occurs prior to E 2.5 of development.

It was shown in fig 3.2 that there is an approximate 80% loss of S100A8 null blastocysts at E 4.5 from S100A8 heterozygous crosses. This indicated an S100A8 null phenotype early in preimplantation development. To determine exactly when S100A8 null lethality occurs it was decided to examine embryos as early as our 2-step PCR technique would allow. To this end embryos at E 3.5 and E 2.5 were generated from S100A8 heterozygous crosses. By

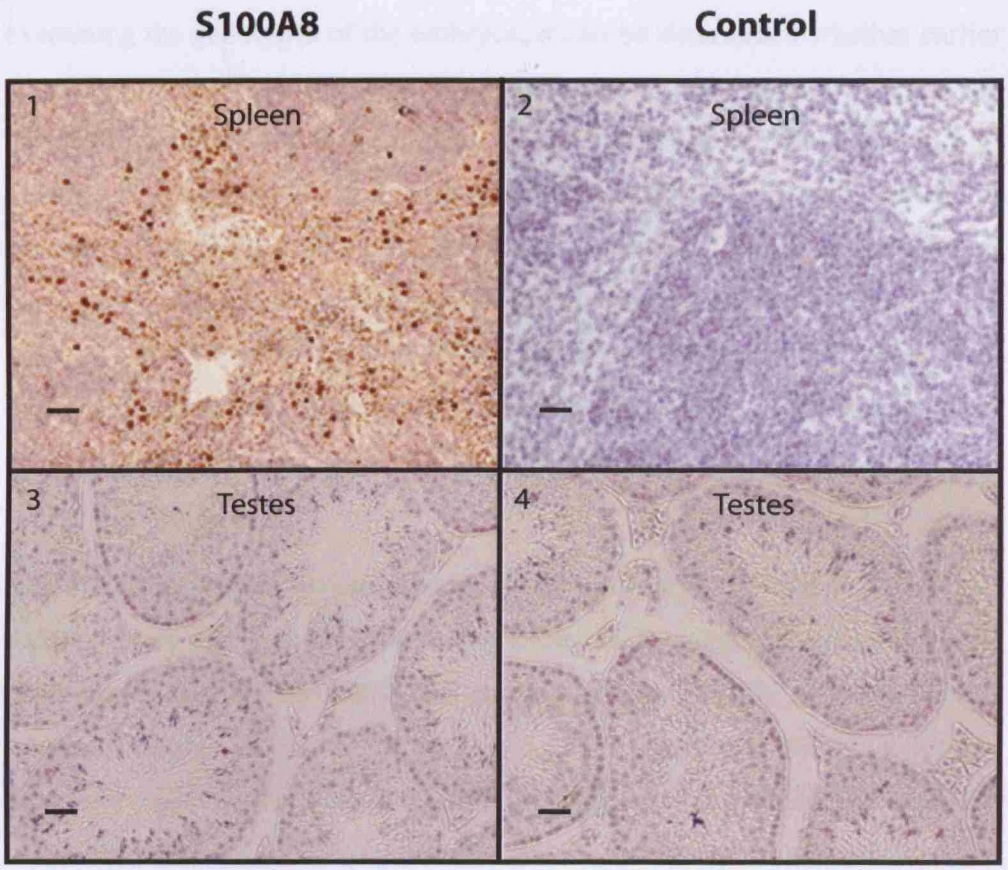


Fig 5.1 S100A8 staining in testes shown by immunohistochemistry. Immunostaining in control spleen tissue sections (1,2) and testes (3,4). Slides show S100A8 with mAb 6A4 staining (1,3) and control mAb staining (2,4). Scale bar = 20μm.

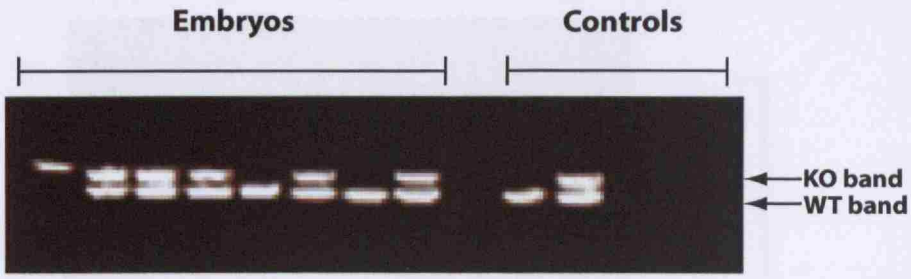
examining the genotypes of the embryos, it can be determined whether earlier stages of embryo show loss of S100A8 null mice or whether null embryos can be seen in expected Mendelian ratios. To determine the stage of S100A8 null lethality it would be expected to see normal S100A8 null embryos present at one stage of development and absent from later stages. It has been determined that some S100A8 null embryos do survive to form blastocysts at E 4.5. It was also seen in C57BL/6J and CD-1 strain mice that there was a higher than expected number of heterozygous blastocysts and so it is of interest if this is also seen at earlier stages. The results are shown in fig 5.2.

S100A8 heterozygous crosses produced embryos at E 3.5 and E 2.5. These embryos were digested and analysed by 2-step PCR using appropriate primers. Embryos at both E 3.5 and E 2.5 showed reduced levels of S100A8 null embryos similar to the result at E 4.5. The resultant ratio of wildtype, heterozygous and null embryos was not the expected 1:2:1 Mendelian ratio but in whole numbers 1:3:0. Again we saw an increase in the numbers of heterozygous embryos and a significant loss of null embryos. Looking at all stages of embryo development analysed, there was an average approximate 80% loss of S100A8 null embryos. This pattern was consistent across 3 days of preimplantation development.

5.2.3 Genotyping of S100A8 heterozygous crosses at E 1.5 is inconsistent.

Having determined that the S100A8 lethality occurs prior to E 2.5, it was decided to investigate embryos at E 1.5. This stage is comprised of embryos at mainly the 1, 2 and 4-cell stages and was believed to be the probable time of S100A8 null lethality. There was concern that the two-step PCR protocol may have difficulty at this level of genetic material, as it is effectively performing PCR on as little as 2-4 DNA strands. As the protocol had worked effectively at E 2.5, it was decided to analyse E 1.5 embryos. Lethality could be detected by normal levels of S100A8 null embryos at E 1.5 with known loss of S100A8 null embryos at E 2.5. The results are shown in fig 5.3.

A



B

Stage	+/+	+/-	-/-	Total
E 3.5#	9	31	1	41
E 2.5#	10	30	3	43

Fig 5.2 Genotyping of preimplantation embryos generated by S100A8 heterozygous crosses. PCR of blastocysts from an S100A8 heterozygous crosses (A) with genotyping results shown in table format (B). #= Cross showed significant deviation from expected genotype ratios $p < 0.01$.

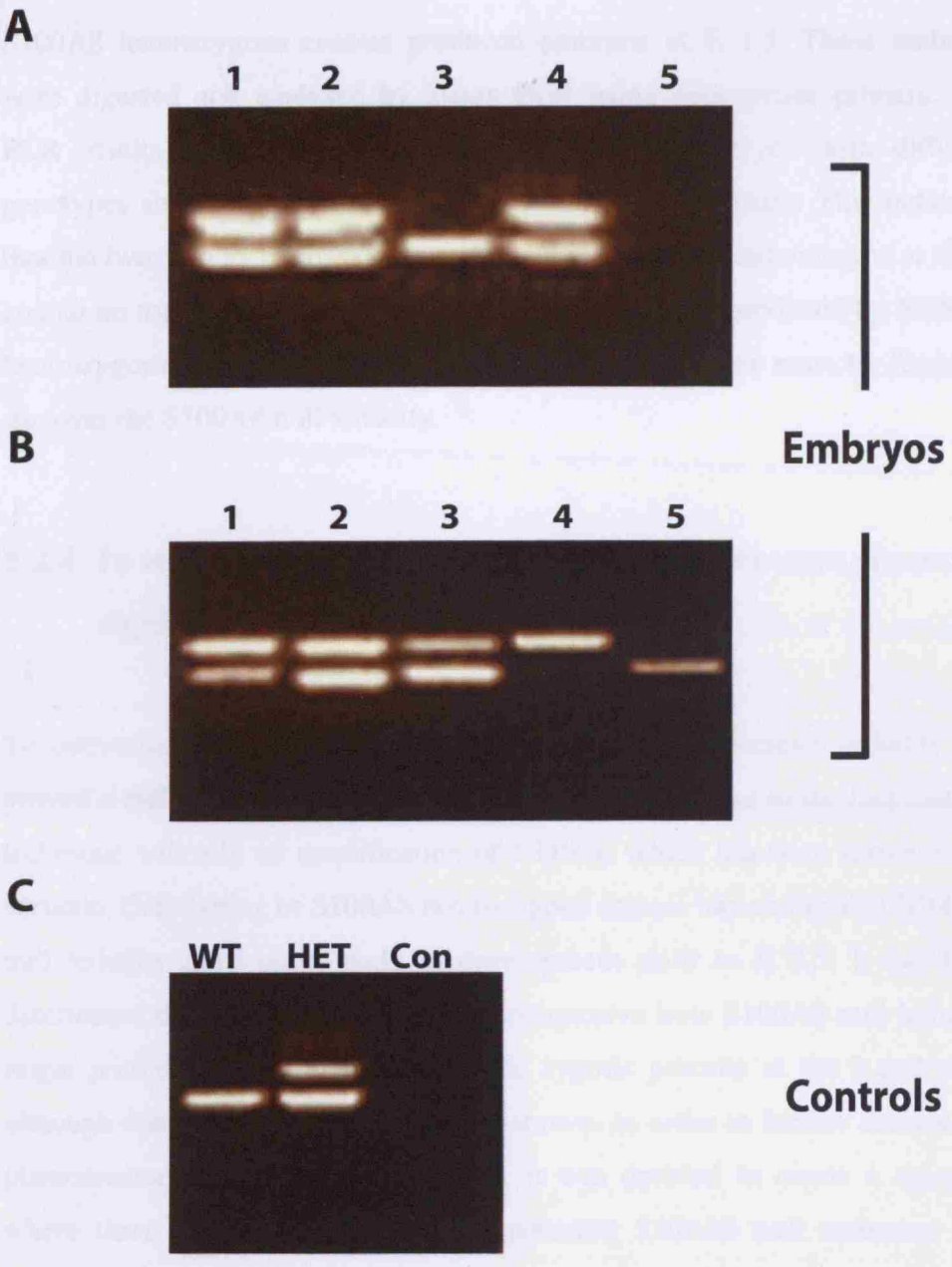


Fig 5.3 PCR analysis of E 1.5 embryos shows inconsistent results.
Repeated PCR of E 1.5 shows that the first reaction results of PCR (A) on 5 selected blastocysts (1-5) differ from the repeat reaction (B). Controls are shown (C) to validate reaction.

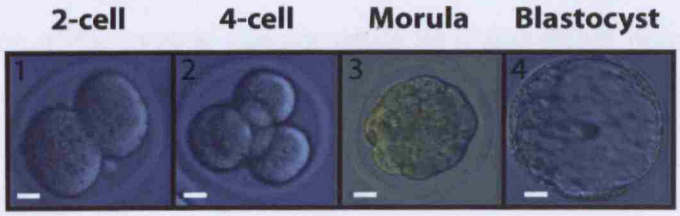
S100A8 heterozygous crosses produced embryos at E 1.5. These embryos were digested and analysed by 2-step PCR using appropriate primers. The PCR results showed inconsistent genotyping for embryos with different genotypes shown for the same embryo in different reactions. This indicated that the two-step PCR protocol is not able to accurately read embryos at E 1.5 and so no true insight can be gained into the genotypes produced by S100A8 heterozygous crosses at this stage. Alternative approaches must be found to discover the S100A8 null lethality.

5.2.4 *In vitro* culture of S100A8 heterozygous crosses shows no significant loss of embryos

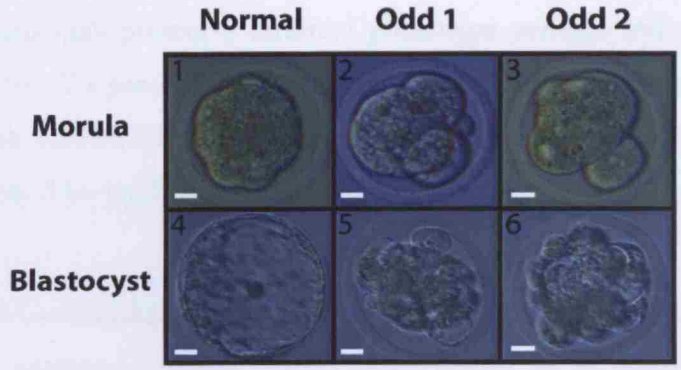
To determine exactly when the S100A8 null embryo presents lethality has proved a difficult task by PCR methods. This could be due to the fact that the technique will rely on amplification of 1 DNA, which has been shown to be variable. Genotyping of S100A8 heterozygous crosses has shown that S100A8 null lethality must occur early in development prior to E 2.5. It has been determined that logically it is difficult to conceive how S100A8 null lethality might present prior to expression of the zygotic genome at the 2-cell stage although this has not been conclusively shown. In order to further analyse the phenomenon of S100A8 null lethality, it was decided to create a scenario where there would be no escape for potential S100A8 null embryos. The problem was that S100A8 null embryos could be rapidly degraded *in vivo* and so not be obtained for genotyping in our experiments. Therefore it was decided to place embryos from S100A8 heterozygous crosses in *in vitro* culture at both 2-cell and 1-cell stages. The main advantage of *in vitro* culture is that it was possible to visualise the embryos as they develop and examine whether their progress is normal. In an *in vitro* system any loss of S100A8 null embryos would be clearly seen and putative embryos would remain within the culture dish to be examined and counted.

The 2 and 1-cell stages were chosen as they occur prior to and at the

A



B



C

Cross	Embryos total	Number blastocysts	Number failed
2-Cell S100A8 heterozygous	42	37	5
wildtype	43	40	2
1-cell S100A8 heterozygous	35	30	5
wildtype	32	28	4

Fig 5.4 *In vitro* culture of embryos from S100A8 heterozygous crosses. In vitro culture of embryos shows normal progression (A) along stages seen in culture (A1-4). Experiments show a population of abnormal embryos from S100A8 heterozygous crosses (B) with normal stage types shown in (B1, 4) and abnormal embryos shown in (B 2-3 & B 5-6). Results of embryo culture are shown in table form (C). Scale bar = 15 μM.

zygotic genome activation, which occurs at the 2-cell stage. It was thought that activation of the zygotic genome could be a significant point to examine for S100A8 null lethality. The 2 and 1-cell stages were also the stages at which our 2-step PCR protocol could not provide accurate genotyping data. Assuming that the S100A8 heterozygous crosses produce Mendelian ratios of fertilised embryos 25% of embryos will be S100A8 null, 50% will be heterozygous and 25% will be wildtype. It could be expected that 25% of embryos may present a different phenotype perhaps indicating S100A8 null lethality. To provide a control for natural loss of embryos within *in vitro* culture C57BL/6J wildtype embryos were also cultured and examined in parallel. The results are shown in fig 5.4.

2 and 1-cell embryos from S100A8 heterozygous crosses along with embryos from wildtype, control crosses were placed in *in vitro* culture until they reached the blastocyst stage approximately 52 hours (2-cell) or 72 hours (1 cell) later. Embryos were analysed at 10am and 5pm on each day of culture and photographs taken. Notes were taken as to the appearance and developmental stage of each embryo. The control wildtype group at both 2-cell and 1-cell showed low loss of embryos (2/43 and 4/32 respectively). All wildtype embryos that failed to progress to the blastocyst stage showed developmental arrest at the 2-cell stage. Having arrested they did not appreciably develop further and looked apoptotic at the end 52/72 hour stage. In both the 2 and 1-cell S100A8 heterozygous cross group a similar low loss of embryos was seen (5/42 and 5/35 respectively). There was no significant numerical difference between the heterozygous and wildtype crosses at either the 2 or 1-cell stage. The S100A8 heterozygous crosses showed a total of 10 embryos from both 2 and 1-cell that did not reach the blastocyst stage. Nine of these embryos arrested at the 4-cell stage and gave a distinctly unusual appearance as shown in fig 5.4. They failed to develop past 4 cells but did undergo compaction to give the unusual shapes shown. All 10 embryos looked apoptotic by the end point of the experiment. The genotypes of these embryos were not known at that stage. There is certainly no evidence that 25% of embryos are lost when S100A8 heterozygous crosses are cultured *in vitro*

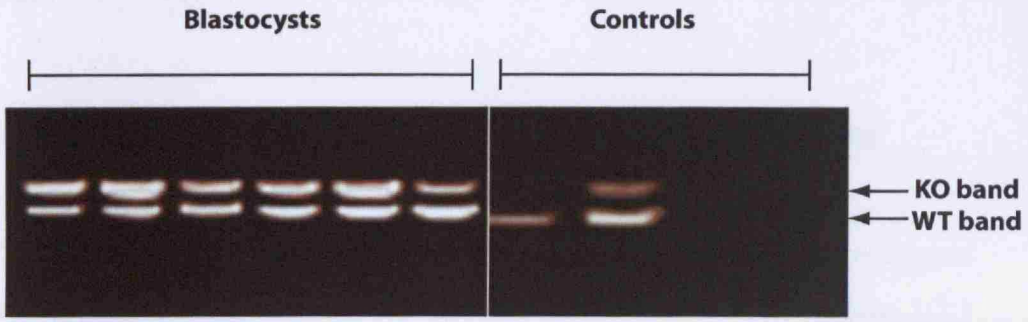
from either 2 or 1-cell embryos, which was an expected result.

5.2.5 Genotyping of cultured embryos shows no S100A8 null embryos at blastocyst stage

While the phenotypes of the embryos did not show any indication of the 25% S100A8 null loss, the genotypes of the embryos were unknown and were of interest. The genotypes of the embryos that arrested were also of considerable interest if this could be determined. It was possible that having been cultured from early embryonic stages that S100A8 null embryos had survived to the blastocyst stage without significant changes in morphology. This would conflict with earlier genotyping data from E 4.5, 3.5 and 2.5 embryos collected *in vivo*, but was examined as a possibility. *In vitro* culture may have supported the S100A8 null embryos or they may not have been destroyed, as they surely would have been *in vivo*. *In vitro* culture might have maintained the S100A8 null phenotype and this possibility needed to be examined. In order to do this the embryos from the S100A8 heterozygous cross *in vitro* culture experiment were genotyped along with embryos from the wildtype crosses *in vitro* culture as a control. The results are shown in fig 5.5.

Embryos from wildtype and S100A8 heterozygous crosses were cultured *in vitro* as detailed in 5.2.4 before snap freezing at the end blastocyst stage. Embryos were digested and genotyped using the 2-step PCR protocol and appropriate primers. Genotyping showed that the wildtype crosses produced 100% wildtype embryos at the blastocyst stage whether generated from 2 or 1-cell stages. Wildtype embryos that showed arrest during *in vitro* culture were wildtype by genotyping. S100A8 heterozygous crosses produced no S100A8 null embryos, a lower than expected number of wildtype embryos and a greatly increased number of heterozygous embryos. The ratio of S100A8 wildtype, heterozygous and null was approximately 1:7:0 from both 2 and 1-cell cultures. Genotyping of S100A8 heterozygous cross embryos that stalled in *in vitro* culture showed that all those embryos were heterozygous. The results of this analysis were highly unexpected and differed sharply

A



B

Cross	+/+	+/-	-/-	Embryos total
2-cell S100A8 heterozygous#	5	35	0	40
wildtype	15	0	0	15
1-cell S100A8 heterozygous#	4	31	0	35
wildtype	15	0	0	15

Fig 5.5 Genotyping of *in vitro* cultured S100A8 heterozygous crosses. PCR genotyping of embryos generated by C57Bl/6J wildtype and S100A8 heterozygous crosses that had been cultured in vitro to blastocyst stage. Sample results are shown (A) and results are presented as a table (B). #= Cross showed significant deviation from expected genotype ratios $p < 0.001$.

from experiments genotyping embryos at E 4.5, 3.5 and 2.5. The unusual result prompted careful analysis of the mice used in the S100A8 heterozygous cross.

5.2.6 Anomalous patterns of S100A8 heterozygous births

The genotyping results of cultured embryos from S100A8 heterozygous crosses shown in fig 5.5 were unexpected. The expected heterozygous 1:2:1 ratio for Mendelian crosses was not seen. A ratio of 1:7:0 was seen with a hugely disproportionate number of heterozygous embryos. An analysis of the mice used was deemed necessary and all mice used in the experiments were analysed along with the genotypes of their littermates and their ancestry. It was discovered that some of the mice used to generate the S100A8 heterozygous crosses shared littermates who had become mating pairs. Three breeding pairs showed unusual patterns of genotypes within their offspring. Breeding pairs to generate experimental mice consist of two S100A8 heterozygous mice, which should have produced a 1:2:0 ratio of wildtype, heterozygous and null offspring, with null offspring not brought to term. The three pairs, (18, 17 and 15) all produced heterozygous offspring with a combined total of 60 mice born which is significantly different from expected offspring genotypes as shown in fig 5.6. This result was curious, as all heterozygous offspring could only have come from crosses of S100A8 wildtype with S100A8 null. As there was no evidence that S100A8 null mice reach term this is difficult to explain.

It was not, however, the first instance of all heterozygous offspring seen in the S100A8 null mouse breeding. In 2004 at the second stage of backcross, three heterozygous males were investigated because all of the offspring from crosses with wildtype C57BL/6J were heterozygous. At this point existence of an S100A8 null mouse was deemed to be the most obvious explanation and so investigation of the three males was undertaken. The males were crossed with SV129 wildtype mice, as it was considered possible that an SV129 background might support an S100A8 null mouse to term. These crosses

A

Pair	Offspring total	Number Het	Number WT
18#	20	20	0
17#	23	23	0
15#	17	17	0
Control	23	15	8

B

Generation	Offspring total	Number Het	Number WT
3 males#	17	17	0
3 males x SV129#	22	22	0
F1 cross	88	63	25

Fig 5.6 Anomalous genotyping results of S100A8 heterozygous crosses. S100A8 heterozygous crosses showed 3 pairs of S100A8 heterozygous mice gave anomalous results in the ratio of wildtype (WT) and heterozygous (Het) offspring (A). This case is similar to 3 males crossed with SV129 wild-type females (B). #= Cross showed significant deviation from expected genotype ratios $p < 0.0001$.

produced only S100A8 heterozygous mice and so it was decided to cross the F1 generation to test whether the ability to produce all heterozygous offspring was inherited. Crosses of F1 generation mice produced normal levels of S100A8 wildtype and heterozygous births with no null mice detected as shown in fig 5.6. It was never determined why the three male mice produced all heterozygous offspring and the investigation was terminated as no other mice displayed similar results and it was thought to be an isolated case. It is possible that the phenomenon of three males in 2004 and the three pairs investigated in 2007 may be linked. There could be no direct descent as all mice produced from the three males of 2004 were excluded from the breeding population although it is possible that the phenomenon could have been retained via relatives. There is no clear explanation as to why the phenomenon arises. Analysis showed that no other mice in the time between the two events have presented with a similar phenomenon. It is clearly not a directly inherited phenomenon and both the litter which produced the three males of 2004 and the litters that produced the 2007 pairs show normal levels of S100A8 wildtype and heterozygous mice. This indicates that the phenomenon has arisen in otherwise normal crosses.

The important aspect is to determine whether this has affected the genotyping results presented in this thesis. The first retrospective analysis undertaken was to look at the genotyping results of the *in vitro* culture experiment, where the mice were used. It was found that littermates of the anomalous pairs were used in the S100A8 heterozygous crosses for the experiments in 5.2.4. The offspring of these mice in the genotyping experiment of 5.2.5 were 100% heterozygous. Removal of the offspring of these mice from the analysis removes the anomalous results. In the 2-cell stage S100A8 heterozygous crosses 35 mice were genotyped. With the anomalous offspring included we see a 1:7:0 ratio of S100A8 wildtype, heterozygous and null. Removal of the offspring shows an approximate 1:3:0 ratio similar to that seen in previous experiments. Similar analysis at the 1-cell stage showed a similar result. The anomalous offspring have changed the result of the experiment.

5.2.7 Confocal microscopy studies of developmental stages for S100A8 protein.

Given the difficulties experienced in determining the exact stage of S100A8 null embryonic lethality, it was decided to approach the problem from a different perspective. In order to cause lethality in a null model a protein must be expressed prior to or at the stage of lethality, and so it would be expected that S100A8 protein would be expressed in early wildtype embryos. Probing of wildtype embryos at different developmental stages could reveal S100A8 protein expression at a specific stage. This would allow us to predict the timing of the S100A8 null lethality and may even give insight to S100A8 function if a distinct pattern of cellular localisation was seen. It was decided to examine S100A8 protein expression by Ab staining using confocal microscopy. The stages initially examined were morula and blastocyst, which were easily available and would provide multiple cells types to examine. The results are shown in fig 5.7.

Samples of embryos at morula and blastocyst stages were generated from C57BL/6J wildtype crosses and fixed in paraformaldehyde (PFA). Expression of S100A8 protein was analysed using Abs 6A4 (not shown) and calgranulin A. Control embryos were analysed using appropriate control Abs (control mAb for 6A4, goat serum for calgranulin A). No S100A8 expression was shown with Ab 6A4 (not shown) but Ab Calgranulin A showed a distinct pattern of expression at the morula and blastocyst stages. The expression pattern was distinctly granular in nature and at the blastocyst stage appears to be predominantly perinuclear.

5.2.8 Calgranulin A staining co-localises with golgi protein GM-130

Calgranulin A staining patterns in morula and blastocyst showed a granular perinuclear expression, which was observed to be similar to golgi proteins at

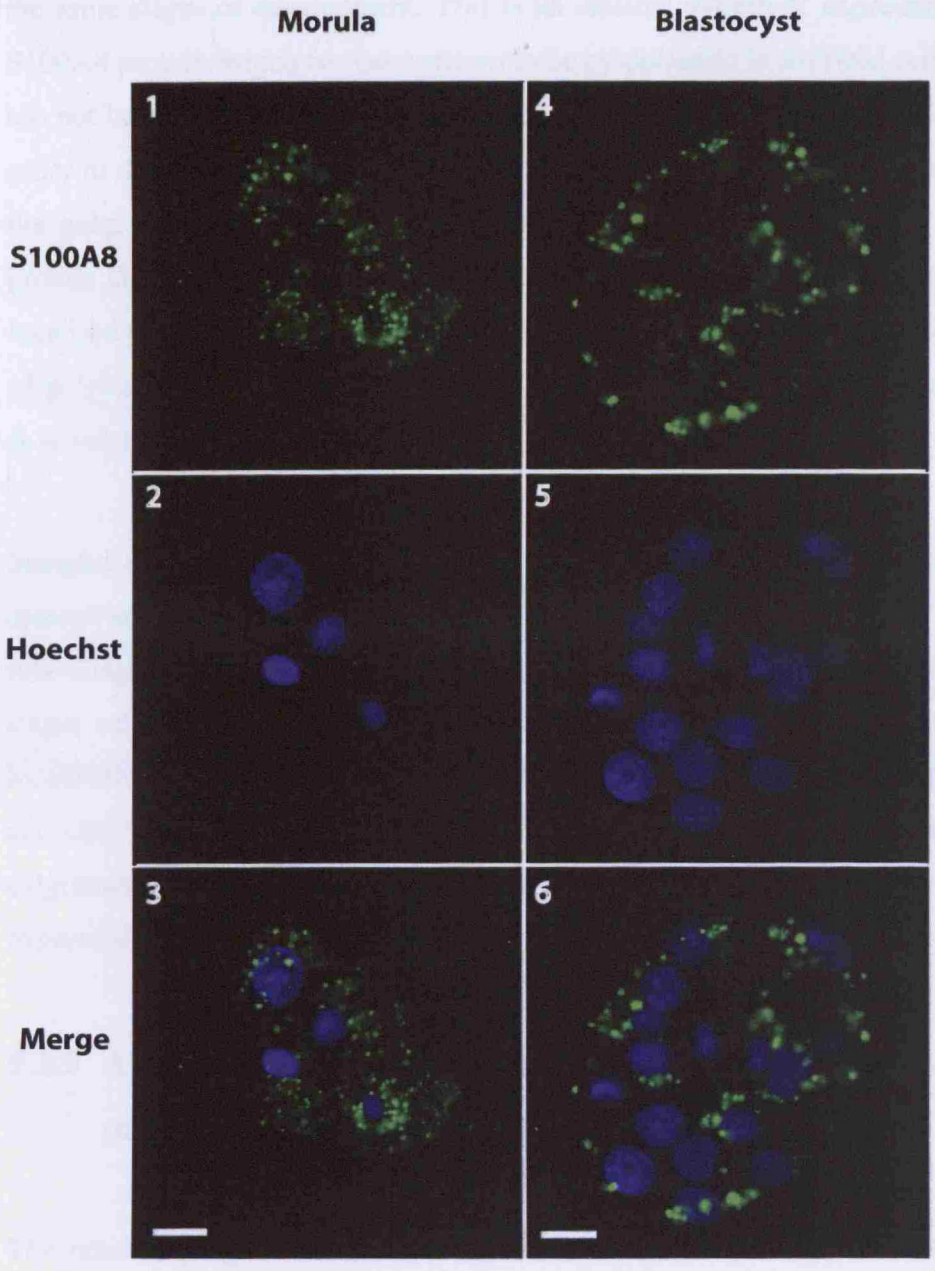


Fig 5.7 Confocal staining of S100A8 in morula and blastocyst. Immunostaining of S100A8 with Ab calgranulin A (1,4), nuclear Hoechst stain (2,5) and merged images (3,6). Panels show morula (1,2,3) and blastocyst (4,5,6) stages. Scale bar = 15 μM.

the same stages of development. This is an unusual pattern of expression for S100A8 protein, which has been shown to be cytoplasmic in myeloid cells and has not been demonstrated to be expressed in the golgi in any study to date. In order to determine whether the calgranulin A staining was indeed localised to the golgi, a double Ab stain was performed with calgranulin A and golgi protein GM-130. GM-130 is a member of the golgin family and known to be localised to golgi and for the purposes of our experiments is used as a marker of golgi expression. Co-localisation analysis would show whether calgranulin A is indeed staining in the golgi. The results are shown in 5.2.8.

Samples of embryos at all stages were generated from C57BL/6J wildtype crosses and fixed in PFA. Expression of S100A8 protein was analysed using Abs Calgranulin A and GM-130 with appropriate serum as controls. At all stages calgranulin A and GM-130 showed considerable but not complete co-localisation. It is clear that there are granular areas with both calgranulin A and GM-130 staining (yellow). It is also clear that there are granules where calgranulin A alone (green) is expressed and others where GM-130 alone is expressed (red).

5.2.9 Ab NH9 staining in developmental stages shows different pattern to Ab calgranulin A staining

The unusual golgi staining pattern given by calgranulin A raised concerns about whether this result truly reflected S100A8 protein staining in preimplantation embryos. It has been reported that S100A8 and S100A9 are seen in primary and secondary granules in myeloid cells (Stroncek, Shankar et al. 2005) but this conflicted with work within our lab showing a cytoplasmic distribution for the proteins (Edgeworth, Gorman et al. 1991). In order to determine whether the Calgranulin A staining was accurate it was decided to retry Ab 6A4 (not shown) and also try Ab NH9, which successfully detects S100A8 by western blot. If calgranulin A was truly showing S100A8 protein expression the Abs should show agreement in their staining pattern. If calgranulin A is giving a false stain then other Abs would give

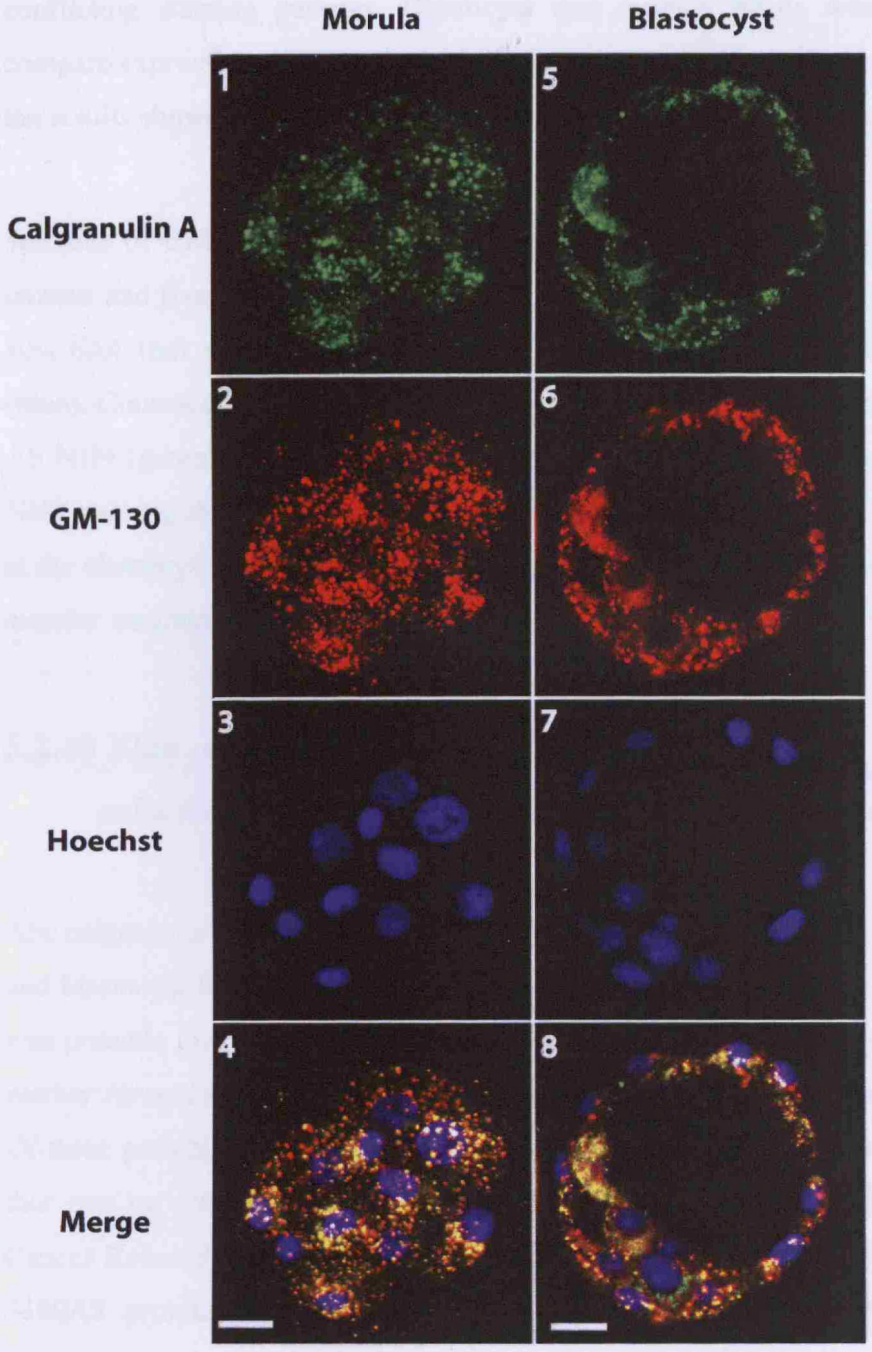


Fig 5.8 Calgranulin A and GM-130 staining in morula and blastocyst. Immunostaining of S100A8 with Ab Calgranulin A (1,5), golgi protein GM-130 (2,6) nuclear Hoechst's stain (3,7) and merged images (4,8) in morula (1-4) and blastocyst (5-8). Scale bar = 20μM.

conflicting staining patterns. Blastocyst and morula stages were used to compare expression with established calgranulin A staining at these stages and the results shown in fig 5.9.

Samples of embryos at all stages were generated from C57BL/6J wildtype crosses and fixed in PFA. Expression of S100A8 protein was analysed using Abs 6A4 (not shown) and NH9 with nuclear staining using Hoechst stain (blue). Control embryos were probed using rabbit serum in place of NH9 Ab. Ab NH9 (green) gave a different pattern of expression to Ab calgranulin A. NH9 staining shows a diffuse cytoplasmic and strong nuclear staining pattern at the blastocyst and morula stage. In both stages the expression is enriched to circular structures within the nucleus, similar to nucleoli morphology.

5.2.10 Flow cytometry of wildtype and S100A9 null myeloid cells shows false positive staining from Ab calgranulin A

Abs calgranulin A and NH9 both gave distinct expression patterns in morula and blastocyst for S100A8. However the patterns were mutually exclusive. It was possible that either calgranulin A or NH9 was truly specific for S100A8, neither Ab was specific or each Ab was recognising different form of S100A8. Of these possibilities it was considered that the most likely explanations were that one or neither Ab was S100A8 specific. NH9 was generated by the Cancer Research UK antibody service raised against full-length recombinant S100A8 protein. Calgranulin A is a commercial Ab (Santa Cruz) raised against a C-terminal S100A8 peptide of 18 residues. It was decided to test both Abs by an alternative technique to see whether either would falsely stain for S100A8. There existed an elegant control model in the S100A9 null mouse. Myeloid cells from wildtype mice are positive for both S100A8 and S100A9 protein, whereas myeloid cells from S100A9 null mice show no expression of S100A8 or S100A9 protein (Hobbs, May et al. 2003). Tests of the two myeloid cell types could show whether either calgranulin A or NH9 falsely stains the S100A9 null myeloid cells. As neutrophils have extensive golgi and nuclei, both staining patterns could be examined. Flow

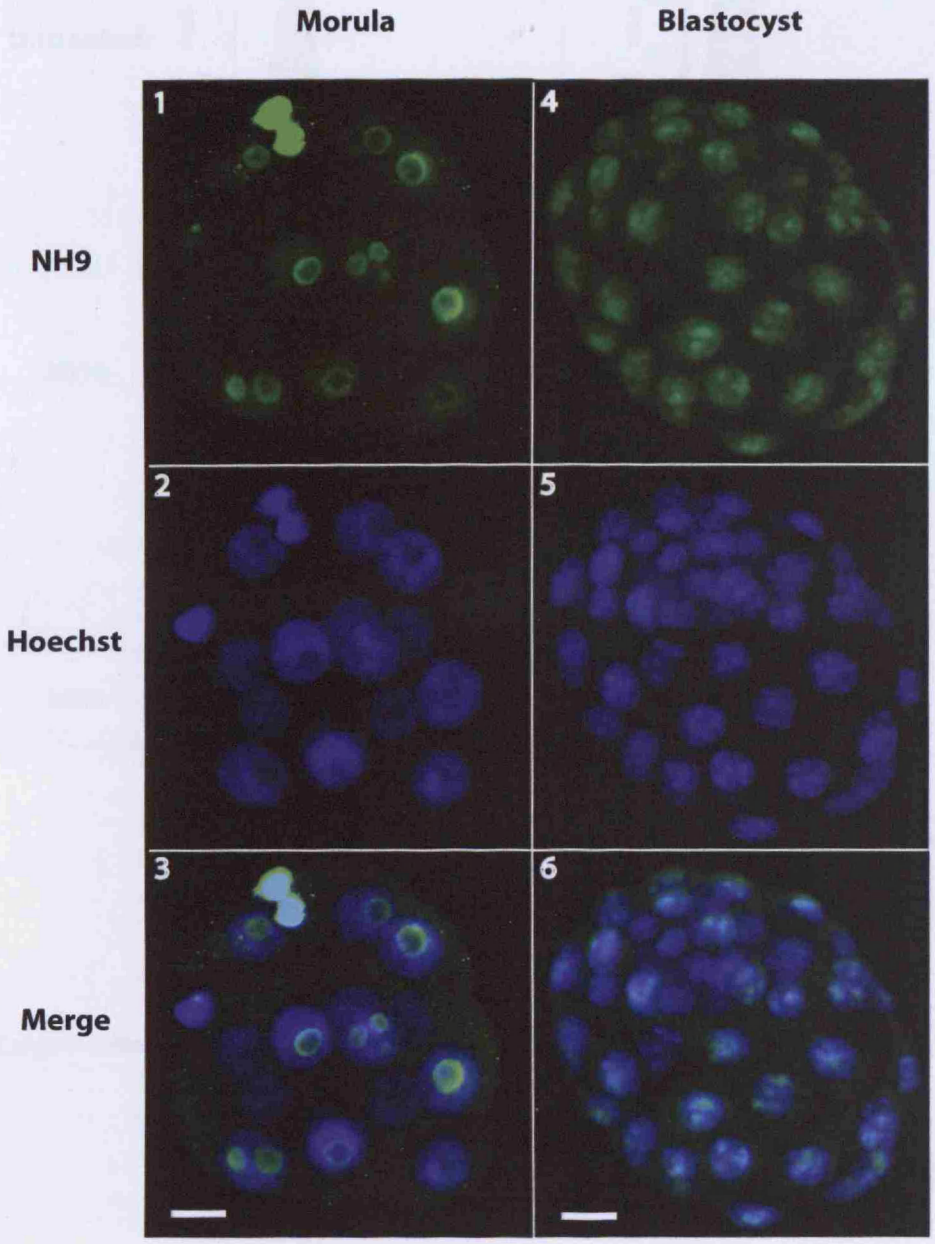


Fig 5.9 NH9 immunostaining in morula and blastocyst. NH9 immunostaining (1,4) and Hoechst staining (2,5) with combined staining (3,6). Staining is shown in morula (1,2,3) and blastocyst (4,5,6). Scale bar = 20 μM.

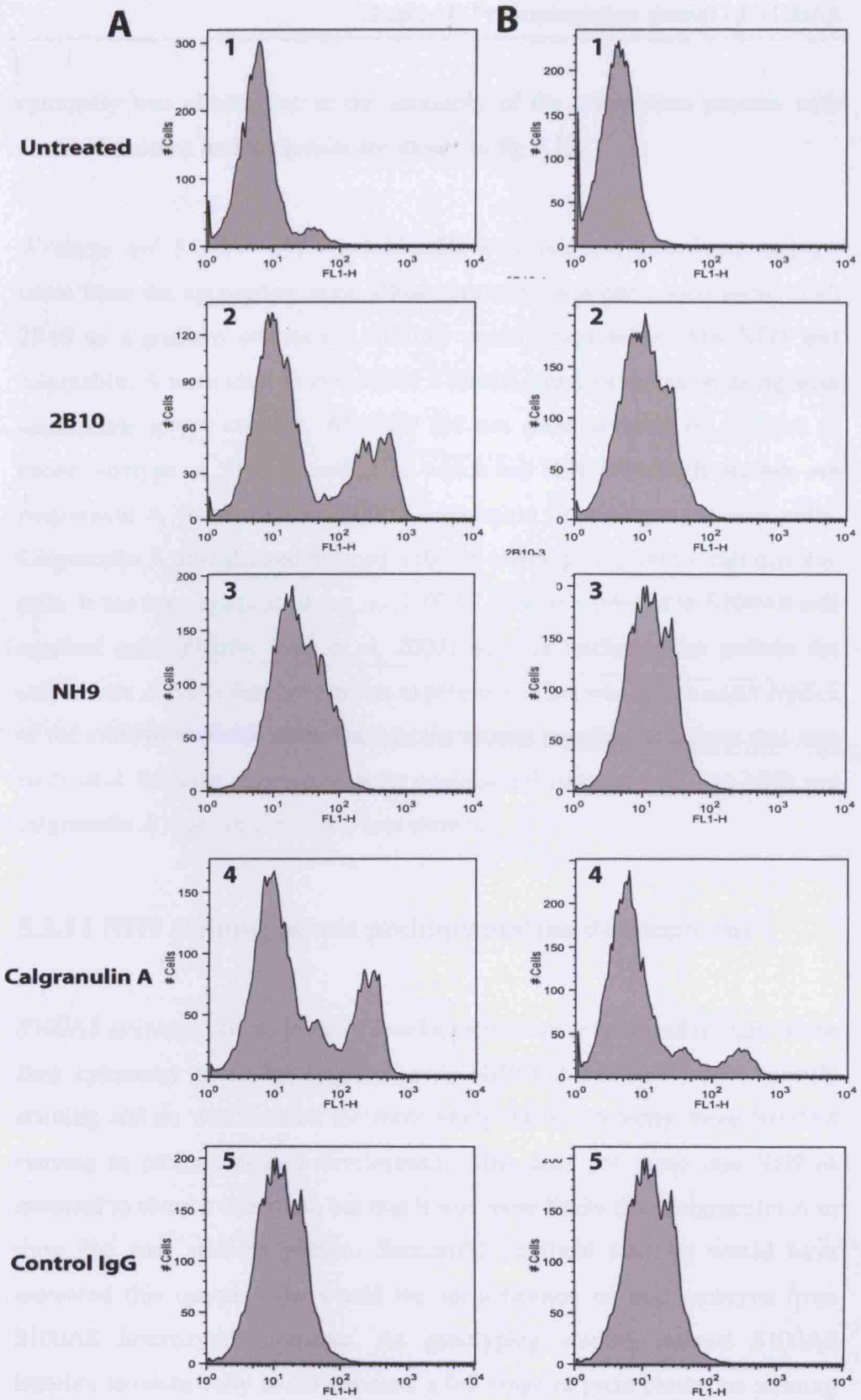


Fig 5.10 Flow cytometry of wildtype and S100A9 myeloid cells. Flowcytometry in wildtype (A) and S100A9 null (B) myeloid cells. Cells were untreated (A1, B1), treated with S100A9 mAb 2B10 (A2, B2), S100A8 Abs NH9 (A3, B3) and calgranulin A (A4,B4) and control IgG (A5, B5).

cytometry was chosen due to the similarity of the preparation process with confocal staining and the results are shown in fig 5.10.

Wildtype and S100A9 null myeloid cells were isolated from bone marrow taken from the appropriate mice. Flow cytometry was performed using mAb 2B10 as a positive control for S100A9 protein expression. Abs NH9 and calgranulin A were used to investigate S100A8 protein expression along with appropriate serum controls. Ab NH9 did not show staining of S100A8 in either wildtype or S100A9 null cells, which has been previously shown. Ab calgranulin A showed clear S100A8 expression in wildtype myeloid cells. Calgranulin A also showed reduced S100A8 staining in S100A9 null myeloid cells. It has been established that no S100A8 protein is present in S100A9 null myeloid cells (Hobbs, May et al. 2003) so it is unclear what protein the calgranulin A Ab is detecting in this experiment. This was not an exact replica of the embryo confocal work but was the closest possible technique that was successful. Staining of neutrophils for confocal microscopy with Abs NH9 and calgranulin A was not successful (not shown).

5.2.11 NH9 staining across preimplantation development

S100A8 staining patterns in early development were re-assessed in light of the flow cytometry result for calgranulin A. NH9 has not shown non-specific staining and so was deemed the most likely Ab to correctly show S100A8 staining in preimplantation development. This does not mean that NH9 as assumed to show a true stain, but that it was more likely than calgranulin A to show the true staining pattern. Successful confocal staining would have answered this question, as would the identification of null embryos from S100A8 heterozygous crosses. As genotyping studies showed S100A8 lethality to occur early in development a full range of preimplantation staining was undertaken to show unfertilised oocyte, 2-cell, 4-cell, morula and blastocyst stages as shown in fig 5.11. An analysis could show insight into the mechanism of S100A8 lethality.

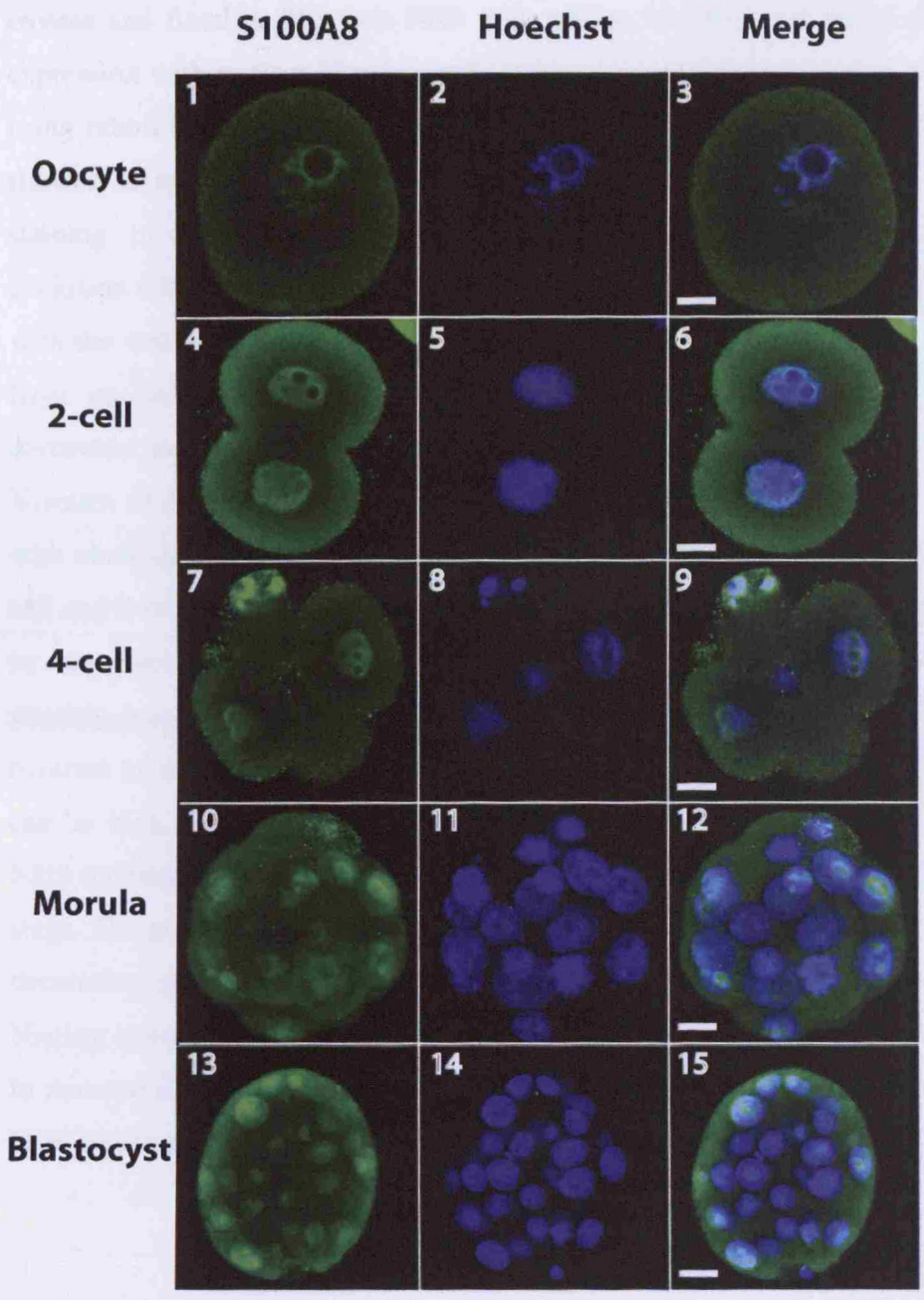


Fig 5.11 S100A8 expression throughout preimplantation development. S100A8 immunostaining in preimplantation development stages. Immunostaining of S100A8 (1,4,7,10,13) nuclear Hoechst stain (2,5,8,11,14) and merged images (3,6,9,12,15). Stages shown are oocyte (1,2,3), 2-cell (4,5,6), 4-cell (7,8,9), morula (10,11,12), blastocyst (13,14,15). Scale bar = 15μM.

Samples of embryos at all stages were generated from C57BL/6J wildtype crosses and fixed in PFA. Ab NH9 was used to investigate S100A8 protein expression with nuclear Hoechst stain in blue. Control embryos were probed using rabbit serum in place of NH9 primary. NH9 staining across all stages showed a nuclear expression confirmed by co-localisation with Hoechst staining. In oocyte, 2-cell and 4-cell the staining showed circular areas of exclusion with no NH9 staining. These areas showed morphological similarity with the nucleolus at these stages. In oocyte the staining of NH9 shows one large nucleolus and was strongly correlated with the Hoechst staining of chromatin and is similar to acetylated histone H4 in chromatin (De La Fuente, Viveiros et al. 2004; De La Fuente 2006). This would indicate localisation with chromatin, which is shown most strongly at the oocyte stage. At the 2-cell and 4-cell stages multiple, circular structures consistent with nucleoli can be seen lacking NH9 staining. In the morula and blastocyst stages NH9 staining was also nuclear but was concentrated within the cell nucleoli in a reversal of earlier exclusion of staining. In these stages some dividing cells can be seen and the chromatin stain of Hoechst is clearly co-localised with NH9 staining. It is not clear whether NH9 was staining in the cytoplasm at any stage. The pattern of expression with high staining at the cell membrane and a decreasing gradient within the cell is indicative of non-specific staining. Nuclear staining within embryonic stages is not commonly observed to be due to non-specific effects of Abs so there was a high degree of confidence that NH9 staining in the nucleus was specific.

5.3 5.3 Discussion

5.3.1 Approaching the S100A8 null phenotype

Data have been presented in this chapter to support a preimplantation lethal phenotype for the S100A8 null mouse, occurring prior to E 2.5 and most likely to occur post fertilisation. It has been shown by embryo PCR that there is significant loss of S100A8 null embryos at E 4.5, 3.5 and 2.5 with an average 80% reduction from expected numbers at each stage. The limit of the 2-step PCR reaction was reached at E 1.5 where genotyping became inconsistent. Examining gamete function S100A8 was not expressed in mouse testes at any stage of sperm differentiation as shown by immunohistochemistry. Experiments to determine the exact timing of S100A8 null embryonic lethality produced mixed results. *In vitro* culture of 2 and 1-cell embryos from S100A8 heterozygous crosses showed no significant loss of embryos although this result was tempered by irregularities in the genotyping of these embryos after culture. Genotyping discovered that an anomaly had arisen within the mouse line that resulted in some mice giving all heterozygous offspring from heterozygous crosses. Confocal staining showed a pattern of expression for Ab Calgranulin A, which was co-localised with golgi marker, GM-130. Ab NH9 showed a different pattern of expression with nuclear localisation. The mutually exclusive patterns indicated that one Ab was falsely staining in preimplantation development. Flow cytometry of wildtype and S100A9 null myeloid cells showed cell staining with calgranulin A in S100A9 null myeloid cells where no S100A8 protein is present (Hobbs, May et al. 2003), whereas NH9 did not stain by flow cytometry. Examination of preimplantation stages with NH9 showed nuclear staining at all stages excluded from the nucleoli at oocyte, 2-cell and 4-cell, but concentrated in nucleoli at the morula and blastocyst stages.

5.3.2 Genotyping studies of S100A8 lethality

In chapter 3 of this thesis, evidence was presented to show that no S100A8 null embryos were found postimplantation. There was also a significant loss of S100A8 null embryos at the E 4.5 blastocyst stage with the null embryos that did survive to this point unable to progress through implantation. Data in this chapter show that S100A8 null lethality must occur prior to E 2.5 as genotyping at E 2.5 and E 3.5 showed the same significant loss of S100A8 null embryos. At each stage a small number of S100A8 null embryos survive consistently showing that it is possible for a minority to escape lethality. This evidence suggests that there is a tight window of S100A8 null lethality between fertilisation and the 8-cell stage. This impression was reinforced by the lack of S100A8 expression in testes and the logical knowledge that S100A8 null gametes only cause lethality when they are combined to form a homozygous null zygote.

No conclusions can be drawn about the state of lethality from the *in vitro* culture experiment with S100A8 heterozygous crosses. The anomaly of all heterozygous offspring seen in litters from three heterozygous pairs is not easily explained. The cause, origin and mechanism of the anomalies in the mice used for this experiment are not known. The three males originally presenting the anomaly and the mice used with the *in vitro* culture experiments were not genetically connected and the phenomenon is not inherited by offspring. Analysis has shown that these anomalies did not affect other experiments and it should be noted that the phenomenon is rare within the mouse line occurring in three known mice and six suspected mice across many generations and hundreds of mice. It is not known whether the anomaly is related to the S100A8 gene or is a consequence of the mouse background. It would be desirable to repeat the *in vitro* culture experiment as it might still yield insight into early preimplantation stages although care must be taken to exclude anomalous mice from this experiment.

5.3.3 Confocal studies of S100A8 in preimplantation development

Confocal staining with Abs calgranulin A and NH9 showed two very different patterns of expression in preimplantation development. Calgranulin A showed a granular stain, which co-localised with GM-130. It would be of interest what the staining pattern for calgranulin A in myeloid cells would be as this experiment was not successful. It has not been widely shown that S100A8 occurs in golgi although one report does shows S100A8 and S100A9 in primary and secondary granules in myeloid cells (Stroncek, Shankar et al. 2005). This report does conflict with work from our group, which demonstrates S100A8/A9 to be expressed in the cytoplasm and not in granules (Edgeworth, Gorman et al. 1991). This work is unfortunately misquoted in Stroncek et al 2005 to support a point in conflict with the data. NH9 showed nuclear staining from oocyte through to blastocyst stages. Nuclear staining has been documented in some cases for S100A8 and S100A9. Human S100A9 was shown to stain in the nuclei of squamous epithelial cells in oesophagus, foetal scalp and psoriatic skin (Robinson and Hogg 2000). Confocal staining of myeloid cells also occasionally showed nuclear staining for S100A9 and S100A8 (E.McNeill – unpublished data). While there is reason to doubt the result of calgranulin A staining in preimplantation development that does not mean that NH9 is true because it does not give false positive staining. It would be desirable to conduct western blot analysis on embryo stages to determine whether S100A8 can be seen at the correct Mw. NH9 immunostaining of S100A8 in embryos generated from heterozygous crosses would also be desirable. It is hoped this would highlight S100A8 null embryos. It is known that small numbers of S100A8 null embryos do survive to blastocyst stage and there might be more null embryos at 2-cell and 4-cell stages.

Expression of S100A8 in the oocyte would provide support for the theory that S100A8 null lethality occurs around the maternal zygotic transition where any maternal transcripts or proteins are degraded. The logic of this is that oocytes retain maternal transcripts and proteins even after meiosis gives rise to a single S100A8 null allele oocyte. S100A8 protein could persist in null oocytes, which could also explain why a small number of S100A8 null embryos survive to blastocyst. This group may retain enough S100A8 transcript or protein to survive the maternal zygotic transition but do not

progress past the blastocyst stage. The majority of S100A8 null zygotes may not retain enough maternal S100A8 transcripts and protein after the maternal zygotic transition. This also raises the possibility that rescue of the S100A8 null embryo is possible by providing transcript/protein at a targeted stage, which would be a desirable experiment to conduct. It would be expected that rescued embryos develop at least to the blastocyst stage although they may not progress past implantation. Expression of S100A8 in the oocyte would also make similar analysis of sperm desirable. Analysis of sperm differentiation in testes showed no S100A8 expression, but the protein may not express until sperm are motile and ready to fertilise. The theory that there was not an S100A8 function in gamete cells may be erroneous as it is possible that proteins retained from the heterozygous germ cell stage rescued gametes.

5.3.4 Conclusions and further study

The evidence presented in this chapter indicates a preimplantation lethality for S100A8 null embryos between fertilisation and the 8-cell stage. A minority of embryos survive this event, possibly due to persistence of maternal S100A8 transcripts and protein in the oocyte. Further study is required to strengthen this finding and further show the exact timing and cause of S100A8 null lethality

CHAPTER 6

6 Discussion

6.1 S100A8 in development

In 1999 R.May in the Leukocyte Adhesion Laboratory produced an S100A8 null mouse with the primary aim to examine the adult myeloid cell function of this protein. In the same year Passey et al 1999 showed that the S100A8 mouse was embryonic lethal and proposed that this occurred at E 8.5-9.5, and was caused by a lack of S100A8 positive foetal cells infiltrating the maternal decidua from the ectoplacental cone region at E 7.5. The functions proposed for the infiltrating cells include either a role co-ordinating the maternal immune response, or prevention of oxidation. While characterising the S100A9 null mouse generated in our lab, J.Hobbs showed that S100A8 is expressed in maternal decidual tissue only, suggesting that our mouse model differed from the published report. M.Mathies showed that S100A8 null mice did not survive to E 8.5, reinforcing the differences between our results and the published report. The aims of this thesis were to re-evaluate the S100A8 null mouse model to determine the true phenotype, to analyse the role of S100A8 in postimplantation development and to discover whether S100A8 played a role in preimplantation development. The conclusion of this report is that the S100A8 null mouse is not lethal at E 8.5-9.5 in development. S100A8 has a role in postimplantation maternal decidual biology, where mRNA is expressed through E 7.5 – E 14.5, whereas protein is detectable only at E 9.5 and E 10.5. We then find S100A8 protein but not mRNA in foetal yolk sac cells at E 9.5 and E 10.5 and foetal liver cells at E 11.5 and hypothesise that it is exported across the placenta. S100A8 is expressed throughout preimplantation development in cell nuclei and lack of expression causes

null lethality between fertilisation and the 8-cell stage of development, most probably after the maternal zygotic genome transition.

6.2 Re-evaluation of the S100A8 null lethality

The first aim of this thesis was to evaluate the S100A8 null phenotype to reconcile differences between our findings (Hobbs 03 and unpublished data from J.Hobbs and M.Mathies) with the published report of Passey et al 1999. S100A8 null embryonic lethality was an unexpected result (Passey, Williams et al. 1999) and was not investigated further despite many unanswered questions. The S100A9 null mouse proved grossly normal (Hobbs, May et al. 2003) and no other reports had shown a function or even expression for S100A8 prior to E 12.5. This was the first report of an S100 family member causing lethality in a null model with the other mouse models showing largely predictable phenotypes given reports into their *in vitro* and *in vivo* functions. Evidence has been presented in this thesis that the S100A8 null lethal phenotype is not as reported but caused by early, preimplantation developmental events between fertilisation and the 8-cell stage.

This thesis has established lines of evidence to justify re-evaluation of the S100A8 lethal phenotype. In agreement with Hobbs et al 2003 data were shown for a maternal decidual expression of S100A8 mRNA, although across a wider period of development than was previously investigated (Passey, Williams et al. 1999; Hobbs, May et al. 2003). This maternal mRNA expression is not universally translated to S100A8 protein showing at least two levels of control for the gene. At E 9.5 and E 10.5 S100A8 is translated in the maternal decidua, showing protein expression for the first time in this tissue. No evidence of foetal expression of S100A8 was shown in this thesis to support null lethality postimplantation. A major justification of postimplantation lethality in the S100A8 null mouse was the presence of S100A8 mRNA expressing cells "infiltrating" the maternal decidua from the embryo at E 7.5. In this thesis evidence has been presented that 7/4 positive cells, most likely to be neutrophils, can surround the embryo at E 7.5.

Neutrophils are attracted to this area to phagocytose apoptotic cells from the decidual reaction (Abrahamsohn and Zorn 1993; Brandon 1993; McMaster, Dey et al. 1993). It is known that neutrophils and monocytes, which both express 7/4 antigen, are positive for S100A8 and S100A9 mRNA and protein. This provides a possible explanation for the "infiltrating" cells and is a difficult question to answer when justifying an S100A8 null lethal phenotype in postimplantation. The infiltrating trophoblast derived cell theory of S100A8 null lethality is further undermined by the fact that the tetraploid aggregation experiment, which should have rescued a null embryo from trophoblast derived lethal defects, did not rescue the S100A8 null mouse.

Studies within the developing embryo showed that S100A8 protein is found in yolk sac cells at E 9.5-10.5 and foetal liver cells at E 11.5. This occurs without its myeloid cell partner, S100A9, and constitutes a novel expression for S100A8. No mRNA could be seen in these cells at any stage by any technique used, which makes it difficult to justify a foetal expression of S100A8. Even in foetal liver where S100A8 and S100A9 are expressed in myeloid lineages the expression pattern for S100A8 is earlier and more extensive than previously shown (Morris, Graham et al. 1991; Lichanska, Browne et al. 1999) and certainly earlier than the proposed lethality in Passey et al 1999. With no data to support expression of S100A8 in foetal tissues prior to E 9.5 and evidence that expression at that stage may not originate from the foetus, it is difficult to support a null lethality caused by lack of S100A8 expression. Two possibilities remain to support this theory. One is that despite extensive study, S100A8 mRNA was expressed in foetal tissues and not discovered. The second possibility is that fundamental differences exist between the mouse models of Passey et al 1999 and our group. However, crossing our mice onto the CD-1 background used in Passey et al 1999 supported the results in our C57BL/6J mice.

6.3 Postimplantation role of S100A8

Having re-evaluated the role of S100A8 protein in postimplantation

development, the conclusion was that the S100A8 null mouse did not present a postimplantation lethal phenotype. It became clear that S100A8 has a role in maternal decidual biology, which would not explain our null model but was of considerable interest. The expression of S100A8 mRNA across at least 7 days of murine development, with protein stably expressed at 2 days indicated at least two levels of control for the gene. It is possible that co-ordinated signals may be required for S100A8 protein expression, with signals deriving either from the maternal tissues, foetal tissues or a combination of both. The pattern of mRNA and protein expression could suggest a "trigger" mechanism for S100A8, with mRNA standing ready for rapid translation to protein upon a further signal. The expression of S100A8 at E 9.5 is possibly indicative of a development within the embryo, perhaps the onset of circulation and the connection of developing circulatory vessels, which would be required for exported S100A8 protein to reach the yolk sac. Further study would be required to discover the genetic regulation of S100A8 and whether distinct transcription factor binding sites related to pregnancy factors such as oestrogen, progesterone or decidualisation cytokines exist. Examining the signals involved in S100A8 decidual expression could prove fruitful in determining the role of the protein in postimplantation development.

The evidence for the model proposed in chapter 4, S100A8 protein export from decidual tissues to foetal yolk sac cells, is important in determining the function of S100A8 in postimplantation development. It was not shown in this study whether S100A8 protein was present in the circulation of the foetus at E 9.5 and E 10.5. This would be experimentally difficult but could be important to demonstrating S100A8 function. It is highly likely that foetal circulation is positive for S100A8 protein at E 10.5 and E 11.5. It was not possible to show whether S100A8 uptake by the yolk sac cells was deliberate or an inevitable consequence of S100A8 presence in the circulation. This is a critical point as the function of S100A8 could be performed in the circulation, in yolk sac cells or a combination of both. This hypothesis could be tested by analysis of the circulation fluid by techniques such as mass spectroscopy or 2D SDS-PAGE, which has yielded good results for proteins exported from cells (Katz-Jaffe, Schoolcraft et al. 2006; Hathout 2007). These approaches should

also provide the Mw measurement matching S100A8 to provide surety of protein identity and perhaps even identify whether the protein is oxidised (Harrison, Raftery et al. 1999). These experiments would be technically difficult but could provide crucial information to support or refute the S100A8 export model.

Possible functions for S100A8 must take into account localisation, timing and the export model. Maternal decidual expression of S100A8, which we propose is then exported across the placenta to foetal circulation and/or cells, implies both foetal and maternal interests are served by S100A8 protein. The possible role of S100A8 could be to co-ordinate functions across maternal and foetal tissues, to protect the foetus or to transport essential material across the placenta. Specific examples of possible S100A8 functions include protection from oxidative damage (Harrison, Raftery et al. 1999; Passey, Williams et al. 1999), leukotriene function by transportation of arachidonic acid (Kannan 2003) or regulation of the maternal immune system response to decidualisation (Brandon 1993; McMaster, Dey et al. 1993). An approach to examine the function of S100A8 in adult tissues would be to generate a conditional null mouse model. A S100A8 null mother would provide vital insight into the role of the protein in decidual biology. It would show whether S100A8 is required for placental development, healthy development of the foetus or immune regulation between maternal and foetal tissues.

6.4 Preimplantation role of S100A8

Having established a role for S100A8 in maternal decidual biology with potential involvement in foetal yolk sac, the question of null lethality was open for re-evaluation. In this thesis evidence has been presented to support a preimplantation lethal phenotype for the S100A8 null mouse. S100A8 has a non-redundant role between fertilisation and the 8-cell stage, most likely to occur after the maternal zygotic transition. S100A8 may have a role in oocyte and sperm prior to fertilisation, which only causes lethality when two null allele gametes combine to form a zygote incapable of expressing

S100A8 protein. It may be that true, functional S100A8 homozygous null mice are only seen after the maternal zygotic transition. If this is the case then S100A8 may have a function in oocyte development and be continuously expressed throughout preimplantation development. It is, however of vital importance that NH9 staining is confirmed as representing S100A8 protein in the confocal studies presented. Efforts to use NH9 stained myeloid cells by confocal were unsuccessful but should not be abandoned. It is also important to assess S100A8 heterozygous crosses between fertilisation (where null gametes survive to) and the 8-cell stage (where genotyping shows null embryo loss) with NH9 immunostaining looking for null embryos. It could show S100A8 null embryos and/or the loss of oocyte S100A8 at the maternal zygotic transition.

It would also be desirable to conduct controlled silencing of the S100A8 gene in wildtype embryos to examine an exclusively null population *in vitro*. Experiments with RNA interference or morpholinos could show lethality caused by inhibition of S100A8 expression. Morpholinos are increasingly successful at early zygotic stages, as their stability is a significant advantage, although as with all RNA based knockdowns there are potential side effects and penetration can be variable (Heasman 2002), strict controls would be necessary. The results of synthetic silencing could be directly observed and would bypass any problem of embryo genotype, mouse background or difference in null models. Successful knockdown could be checked by NH9 staining and western blot, which should be reduced if the S100A8 gene were silenced.

The expression pattern of NH9 suggests a nuclear expression of S100A8, excluded from nucleolus in oocyte, 2-and 4-cell embryos, but concentrated in the nucleoli at morula and blastocyst stages. The staining pattern of NH9 suggests S100A8 could be associated with chromatin and gene regulation. It is not known why S100A8 would switch expression pattern at the morula and blastocyst stage and accumulate in the nucleoli, although this may indicate a change of function necessary to implantation. One issue that arises from this study is whether S100A8 has multiple functions throughout development.

One, non-redundant function is essential to explain the lethality of the S100A8 null mouse but there may be several stages where S100A8 plays redundant or non-redundant roles. This could explain the differences between the report of Passey et al 1999 and the data presented in this thesis with the published model surviving early lethality. It has been shown that the few null embryos that survive to the blastocyst stage do not progress past implantation. It is not known whether this is because the embryos are fatally compromised despite surviving the early lethality or whether there is a second requirement for S100A8 to survive implantation. This could be determined by rescue of S100A8 null embryos with purified RNA injection at the critical early lethality stage for short-term rescue. Injection of an S100A8 vector, which will stably express mRNA throughout preimplantation development can examine more definitive rescue. Rescued embryos could survive past implantation or fail at the next S100A8 related hurdle. It would be of obvious interest whether rescued S100A8 null embryos could survive past the E 8.5-9.5 period of development.

6.5 Conclusions

This thesis has advanced knowledge in the area of the S100A8 null lethal mouse. The field of the S100 proteins is a difficult one to study with much *in vitro* data and results that differ sharply between groups. The *in vivo* models for both S100A8 and S100A9 are highly insightful to the field illustrating the limitations of *in vitro* data and more subtle complex roles for the proteins. This study has shown that the S100A8 null mouse is embryonic lethal between fertilisation and 8-cell, that S100A8 has an interesting and novel role in maternal decidual biology and that S100A8 can indeed be studied independently of its myeloid partner, S100A9.

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