THE GENERATION OF TEETH FROM MOUSE EMBRYONIC STEM CELLS

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DECLARATION

I, Anita Marais, declare that this dissertation is my own work. It is being submitted for the Degree of Master of Science in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other University.

Anita Marais

3rd day of March 2010

ABSTRACT

Embryonic stem cells are known to differentiate into tooth-like cells when combined with 10-day embryonic oral epithelium. This study aimed to determine whether mouse embryonic stem cells could also contribute to tooth formation when combined with lower jaw ectomesenchyme from mouse embryos. The epithelium and ectomesenchyme of 10,5-day and 12,5-day mouse embryonic lower jaws were separated. The 10,5-day lower jaw epithelium and 12,5-day lower jaw ectomesenchyme were respectively combined with either mouse embryonic stem cells or STO cells as control and cultured *in vitro*. Some of the explants were grown as renal subcapsular grafts for another 21 days. Histological and immunocytochemical analyses were performed on all the explants retrieved. Tooth buds were not identified in the *in vitro* cultures. The stem cell and jaw epithelium, STO cell and jaw epithelium and STO cell and jaw mesenchyme renal subcapsular grafts showed no tooth formation. Four out of ten stem cell and jaw mesenchyme renal subcapsular grafts developed teeth. The stem cells could not be identified after differentiating, but may have contributed to the tooth formation.

In memory of my grandmother

Henriëtta Carolina Booysen

1923 – 2007

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1. INTRODUCTION

Tooth development has been studied in great detail at both the morphological and molecular levels in order to determine the mechanisms involved in forming these complex structures. The formation of teeth is dependant on signalling interactions between the oral epithelium and the cranial neural crest-derived ectomesenchyme (Lumsden, 1988; Baker *et al.*, 1997; Thesleff and Sharpe, 1997; Chai and Slavkin 2003). The use of mouse embryos has proved to be a good model for the study of tooth formation (Miletich and Sharpe, 2003). In mice, cranial neural crest cells migrate into the first pharyngeal arch around the ninth day of embryonic development (Hay, 1961; Chai and Slavkin, 2003). By the tenth day, the mandibular processes join to form an arch (Hay, 1961). The odontogenic thickenings of the presumptive molars become evident in the epithelium from embryonic day ten to eleven (Osumi-Yamashita *et al.,* 1994). At day twelve, these thickenings become invaginated into the underlying mesenchyme to form the dental lamina of the presumptive molars (Osumi-Yamashita *et al.,* 1994; Chai *et al.,* 1998). At day thirteen of development, the first molars are represented by bud-shaped swellings, which form from the dental lamina (Hay, 1961) and this is known as the bud-stage (Kollar and Baird, 1969). At embryonic day fourteen, each molar bud forms a cap. This stage of tooth development is known as the cap-stage (Peters and Balling, 1999). During this stage the enamel organ composed of an inner and outer enamel epithelium, and the dental papilla become evident (Ten Cate *et al.,* 2003). The enamel knot forms from the dental epithelium and becomes the signalling centre that controls further tooth formation (Hay, 1961; Peters and Balling, 1999).

Reciprocal epithelial-mesenchymal interactions are essential for tooth development. Tooth formation is said to be initiated in the epithelium (Baker *et al.*, 1997; Chai and Slavkin 2003). It has been shown that the dental epithelium has the potential to induce tooth formation from as early as the ninth day of embryonic development (Mina and Kollar, 1987; Lumsden, 1988). From the ninth to the eleventh day, the epithelial signalling molecules BMP4 (bone morphogenic protein) and FGF8 (fibroblast growth factor) induce the pluripotent neural crest cells to become dental ectomesenchymal cells (Bei and Maas, 1998; Ferguson *et al.,* 2000; Ohazama *et al.,* 2004; Blentic *et al.,* 2008). FGF8 regulates the expression of *Msx1, Pax9, Lhx6* and *Lhx7* (Bei and Maas, 1998; Grigoriou *et al*., 1998), while BMP4 induces the expression of *Msx1* in the ectomesenchyme (Satokata and Maas, 1994). During the eleventh day of embryonic development, the potentiality of the epithelium shifts to the oral ectomesenchyme (Mina and Kollar, 1987; Lumsden, 1988; Peters and Balling, 1999), where MSX1 and PAX9 regulate the expression of mesenchymal *Bmp4*. Ectomesenchymal BMP4 is involved in the induction of molecules of the dental epithelium, resulting in the transition of the tooth bud to the cap stage (Peters *et al.,* 1998; Thesleff, 2003). If the oral epithelium or ectomesenchyme is removed from the jaw during the time that each of these layers possesses odontogenic potentiality, they are able to induce tooth formation when recombined respectively with mesenchyme or epithelium of non-jaw origin (Mina and Kollar, 1987).

It is important to select embryos of the correct developmental ages when studying the epithelial–mesenchymal interactions that occur during tooth formation, due to the shift in odontogenic potential from the epithelium to the ectomesenchyme. For this reason, mouse embryos of two specific developmental ages were selected for the present study.

Embryos of 10,5 days were chosen to study the inductive potential that is believed to reside in the oral epithelium at this time (Mina and Kollar, 1987; Lumsden, 1988). Embryos of 12,5 days were chosen to study the inductive potential believed to be present in the lower jaw ectomesenchyme from the twelfth day of development (Mina and Kollar, 1987; Lumsden, 1988; Peters and Balling, 1999).

During development, the dental epithelium gives rise to the enamel organ and subsequently to the ameloblasts, while the ectomesenchyme gives rise to the remainder of the tooth, except for the cells lining the blood vessels (Lumsden, 1988; Cobourne and Sharpe, 2003). It has been shown that dissociated cells of the dental epithelium and ectomesenchyme of mouse incisors were able to form normal teeth when reconstituted and cultured *in vitro* and *in vivo* (Nakao *et al.,* 2007). These explants could successfully be implanted into the jaws of adult mice to develop into functional incisors. The cells used in this reconstitution, were taken from the cap-stage of tooth development (E14.5). It may be presumed that the cells had received the necessary inductive signals to form specific structures of the tooth germs by the time they were harvested (Ten Cate *et al.,* 2003).

Embryonic stem cells on the other hand are totipotent cells, competent to become any cell type in the body, provided that they are induced by the correct cascade of signalling molecules. Both embryonic and adult stem cells have been used to form teeth when combined with embryonic oral epithelium (Ohazama *et al.,* 2004; Modino and Sharpe, 2005; Hu *et al*., 2006). In a study carried out by Ohazama *et al*. (2004), it was shown that when adult bone marrow-derived cells, embryonic stem cells or neural stem cells were co-cultured with mandibular epithelium of 10-day old mouse embryos, the stem cells in

all three of these groups expressed the mesenchymal odontogenic markers *Pax9, Msx1* and *Lhx7*, indicating that tooth formation was taking place. In this case, the oral epithelial signalling molecules FGF8 and BMP4 served to initiate a signalling response in all three of the stem cell lines, presumably inducing the stem cells to differentiate into dental ectomesenchymal cells. The dental ectomesenchymal signalling molecules *Pax9* and *Msx1* induce the expression of mesenchymal *Bmp4*. BMP4 in turn induces the progression of tooth formation in the dental epithelium by the activation of enamel knot signalling molecules *p21, Msx2*, *Bmp4* and *Fgf4* (Peters *et al.,* 1998; Thesleff, 2003).

Although 12,5-day mouse embryonic oral ectomesenchyme express all the signals necessary for normal tooth formation to progress, the inductive potential of the oral ectomesenchyme with regards to specifically embryonic stem cells has never before been studied. Provided that the odontogenic potential of the epithelium can effectively switch to the ectomesenchyme during tooth formation, it is possible that stem cells may be induced to form teeth in a similar fashion using either epithelium or ectomesenchyme of the appropriate developmental stages. The aim of this study was thus to determine whether embryonic stem cells were able to differentiate and contribute to tooth formation when combined with mouse embryonic lower jaw ectomesenchyme *in vitro* and *in vivo*.

2. MATERIALS AND METHODS

This study was approved by the Animal Ethics Committee of the University of the Witwatersrand (AEC 2007/27/4).

2.1 Terminology

Throughout the study reference has been made to “explants” and “grafts”. These terms refer to tissues that have been cultured in different ways. In this study the term “explant” refers to the transfer of living tissue from mouse embryos to a suitable artificial environment for *in vitro* culturing. The term “graft” in this study refers to any tissue transplanted to a living host or recipient. All tissue transplanted on to chorio-allantoic membranes of chicken eggs and grown *in ovo*, or implanted under the renal capsules of mouse kidneys and grown *in vivo,* are therefore termed “grafts”.

2.2 STO cell culture

A SIM mouse, 6-thioguanine and ouabain-resistant (STO) fibroblast cell colony (ATCC, Cat.#: CRL-1503) was established from frozen cells. The cells were thawed at 37°C and pipetted into a 10ml centrifuge tube. 5ml of fresh STO-cell medium [Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 4.5g/L glucose, 0.11g/L sodium pyruvate (Ref #: 41966-029, Gibco, Invitrogen), 10% heat inactivated fetal bovine serum (Ref #: 10108-157, Gibco, Invitrogen) and 0,1% pen/strep] (Appendix A) was slowly added to the thawed cells. The cells were centrifuged at 1000rpm for 5 minutes to form a pellet. The supernatant was discarded and the pellet of cells was resuspended in 1ml of fresh STO-cell medium. The cells were plated into a 25cm^2 culture flask (Nunclon, Roskilde, Denmark) along with 5ml of fresh STO-cell medium. The flask of cells was placed in a

humidified incubator at 37°C in air containing 5% CO_2. The cells were grown to confluence ($4,2x10^6$ cells/ml).

After the STO cells had reached confluence, they were subcultured. The medium was removed from each of the flasks. The cells were then rinsed in 2ml of sterile PBS (Appendix B) to remove excess serum. The PBS was replaced with 2ml of 25% trypsin/EDTA (Sigma, T4299) in PBS to detach the cells from the flask. The trypsin/EDTA was inactivated using 5ml of STO-cell medium. The STO cells were decanted into a 10ml centrifuge tube and centrifuged at 1000rpm for 5 minutes to form a pellet. The supernatant was discarded and the cell pellet was resuspended in 3ml of fresh STO-cell medium. One millilitre of the cells was plated into each of three $25cm^2$ culture flasks along with 5ml of STO-cell medium. The cells were cultured in a humidified incubator at 37˚C in air containing 5% CO_2 and once again grown to confluence (Fig. 1).

2.3 STO-cell feeder plate preparation

The STO-cell feeder layers were established by treating confluent STO cells with 2ml of 50mM Mitomycin (Sigma-ALDRICH, Cat #. M-0503) (Appendix C) for 2 hours. The cells were then trypsinized and centrifuged to form a pellet. The supernatant was discarded and the pellet was resuspended in 3ml of fresh STO-cell medium. One millilitre of the cells were plated into each of three $25cm^2$ culture flasks along with 5ml of STO-cell medium. The cells were cultured in a humidified incubator at 37°C in air containing 5% CO_2 until needed (approximately 3 to 4 days).

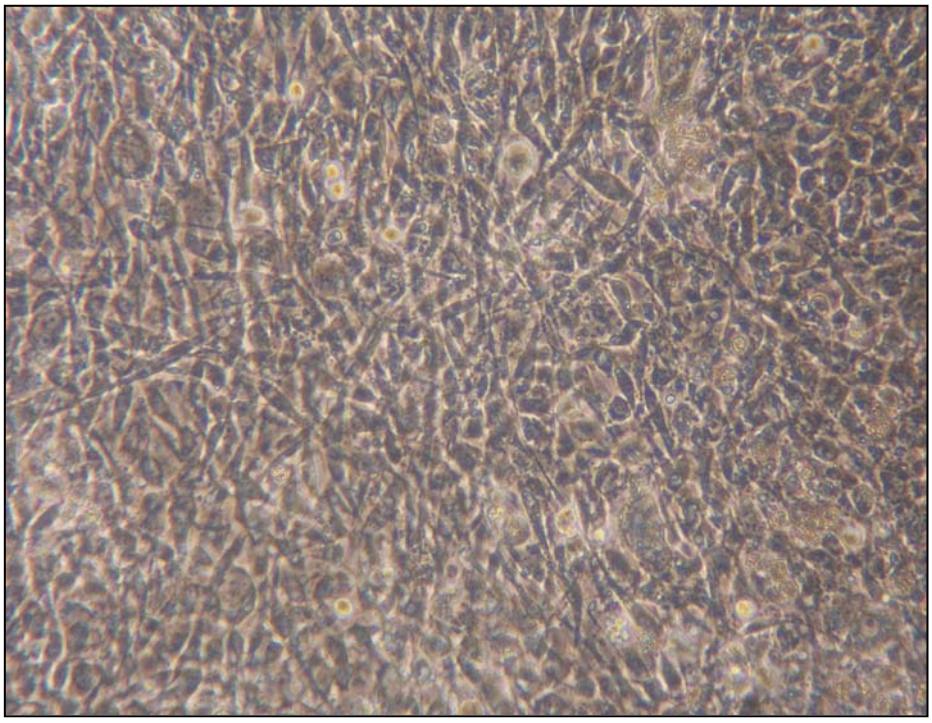

Figure 1: Living STO cells 60 hours after seeding. The cells have grown to confluence. (x 200) (Phase contrast microscopy).

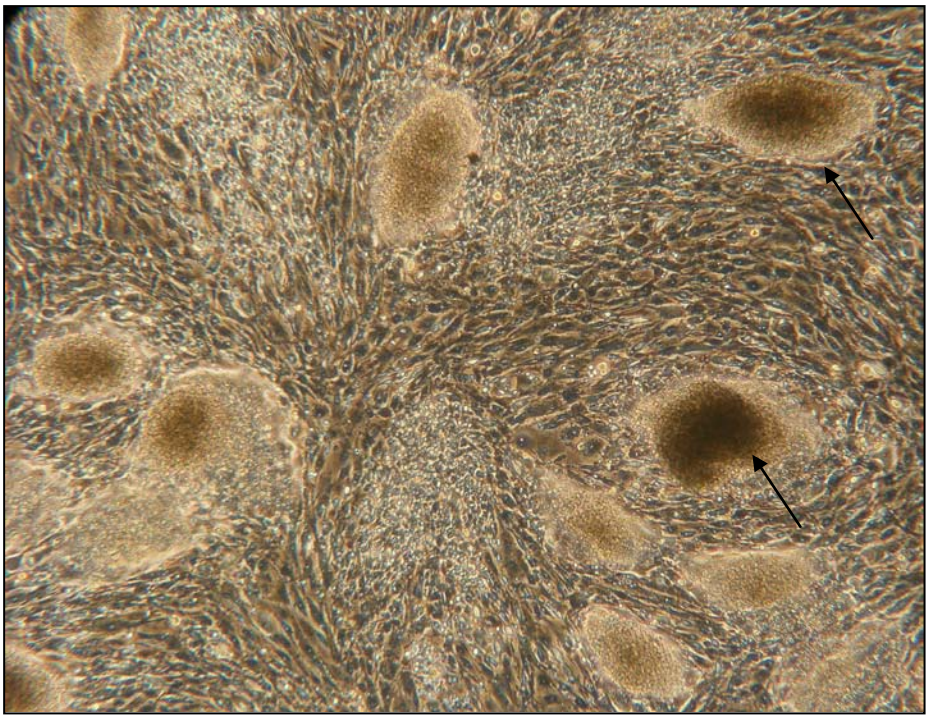

Figure 2: Clusters of murine embryonic stem cells which had been plated on to a prepared layer of feeder STO cells. The two large clusters on the right (arrows) appear to be more dense due to the upward proliferation of the ES cells. (x 100) (Phase contrast microscopy).

2.4 Embryonic stem cell culture

Murine embryonic stem cells were cultured in 25cm^2 tissue culture flasks on the prepared STO-cell feeder layers in embryonic stem cell medium. The embryonic stem cells were obtained from the blastocyst stage of mouse embryos (Strain: 129/Sv+c/+p) (ATCC, Cat.#: CRL-11632). These ES cells were thawed and pipetted into a 10 ml centrifuge tube with 5ml of embryonic stem cell (ES-cell) medium. This medium contains Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 4.5g/L glucose, 0.11g/L sodium pyruvate, 15% heat inactivated fetal bovine serum, 0.1mM β mercaptoethanol, 0,2μM leukemia inhibitory factor (LIF) and 0,1% pen/strep (Appendix D). The ES cells were then centrifuged in the ES-cell medium at 1000rpm for 10 minutes to form a pellet. The supernatant was discarded and the cell pellet was resuspended in 1ml of fresh ES-cell medium. The ES cells were then plated along with 5ml of ES-cell medium into a 25cm^2 culture flask that had been previously seeded with a STO-cell feeder layer. The cells were cultured in a humidified incubator at 37°C in air containing 5% CO_2. Since these ES cells grow as isolated clusters (Fig. 2), the confluence of each flask was determined by the concentration of ES cell clusters, the rate of size increase of the clusters and hence the rate of proliferation of ES cells in the respective clusters. The concentration of ES cells per cluster was measured using a haemocytometer, which gave an indication of the number of clusters that needed to be used for each explant. The number of ES cells per cluster was determined to be approximately 5x10^3 cells/cluster.

A subculturing procedure stipulated by the ES cell product information sheet (CRL-11632, ATCC, U.S.A) was initially followed. The ES-cell medium was discarded and the culture flask rinsed with 0,25% trypsin/EDTA to remove the fetal bovine serum. ES cells

were trypsinized using 2ml of 25% trypsin/EDTA. However, the dispersed ES cells obtained using this method were contaminated with STO cells, which had detached from the surface of the flask during trypsinization. In order to reduce the inclusion of STO cells, an alternate method of separating the ES cells from their feeder layer was developed. The ES cells grow in colonies that appear as clusters on top of the STO cell feeder layer (Fig. 2) (Johkura *et al.,* 2004). The ES cells could therefore easily be collected by manually scratching the individual ES cell clusters off the STO-cell feeder layer using a fine sterile needle. These clusters of cells were then pipetted into a 10ml centrifuge tube and centrifuged at 1000rpm for 1 minute to form a pellet. The supernatant was discarded and the clusters were resuspended in 2ml of 25% trypsin/EDTA (Sigma, T4299) in PBS for 10 minutes to detach the cells from each other. The trypsin/EDTA was inactivated using 5ml of ES-cell medium. The ES cells were again centrifuged at 1000rpm for 5 minutes to form a pellet. The supernatant was discarded and the cell pellet was resuspended in 3ml of fresh ES-cell medium. The ES cells were then plated along with 5ml of fresh ES-cell medium into a 25cm^2 culture flask that had been previously seeded with a STO-cell feeder layer. The cells were cultured in a humidified incubator at 37C in air containing 5% CO_2 and confluence was again determined as described above.

2.5 ES cell cluster and STO cell pellet preparation

The ES cells that were used for the clusters had been passaged at least three times. The ES cell clusters were prepared for culturing in combination with mouse embryonic tissue by scratching the clusters off the STO-cell feeder layer as described previously. However, these ES cell clusters were not trypsinized to form an individual cell suspension. Instead, the intact clusters of ES cells were resuspended in 1ml of fresh

tissue-culture medium (DMEM supplemented with glutamax, 10% heat inactivated fetal bovine serum and 0,1% pen/strep) (Gibco, Invitrogen, Ref #. 31966-021) (Appendix E).

For the preparation of the control STO cell pellets, the STO-cell medium was removed from a flask of STO cells previously grown to confluence. The cells were rinsed in 2ml of PBS, after which they were trypsinized using 2ml of 25% trypsin/EDTA (Sigma, T4299) in PBS until the cells detached from the flask. The trypsin/EDTA was inactivated using 5ml of STO-cell medium. The STO cells were decanted into a 10ml centrifuge tube and centrifuged at 1000rpm for 5 minutes to form a pellet. The supernatant was discarded and replaced with 3ml of fresh tissue-culture medium. The STO cells were suspended into a single cell suspension, after which 500µl of the suspension was pipetted into each of eight Eppendorf tubes. Each Eppendorf tube containing the STO cell suspension was centrifuged to form a pellet. The concentration of cells per pellet was measured using a haemocytometer, which gave an indication of the size of the pellets to be used (approximately $7x10^4$ cells/pellet). The pellet of cells was dislodged from the base of the Eppendorf tube by gently pipetting the medium up and down the inside of the tube.

2.6 Preparation of the embryonic tissue used for the cell-tissue combinations

NMRI X NMRI mouse embryos of 10.5 days and 12.5 days were obtained for this study. Sixteen female NMRI X NMRI mice (*Mus musculus*) were mated at the National Health Laboratory Services (NHLS) and housed in the Central Animal Unit at the University of the Witwatersrand during the course of the study. All experimental procedures involving animals were carried out in the School of Anatomical Sciences, University of the Witwatersrand under aseptic conditions. All instruments and solutions were sterilised/autoclaved before use to prevent contamination.

Pregnant female mice were euthanased via an intraperitoneal injection at 10.5 days (N=7) and 12.5 days (N=9) of pregnancy respectively (Eutha-naze - Centaur Labs, R.S.A., Lot #. 302963). The uterine horns containing the embryos were removed and placed in a sterile glass bowl containing Tyrode's solution (Appendix F) in a humidified incubator at 37°C in air containing 5% CO_2. Each embryo was removed from the uterine horn in turn, placed in a black wax dish with Tyrode's solution and pinned on to its side using fine insect pins (Figs. 3A,B and 4A,B). Both embryonic forelimbs were removed and placed in a glass cavity block with 0,04% collagenase in Tyrode's solution for 10 – 20 minutes (type VII; Sigma Chemical Co., USA, Lot#. 114K8634). Forelimb epithelium and mesenchyme were selected as a control to the lower jaw tissue to show whether epithelial-mesenchymal interactions occur successfully if one of the tissue layers is replaced with cells (Vaahtokari *et al*. 1996). The head of each embryo was removed just caudal to the first pharyngeal arch. Each head was then pinned so that the ventral surface was uppermost (Fig. 3C and 4C). The lower jaw (10,5-day or 12,5-day) was carefully removed using a fine ophthalmic knife. A transverse view of the lower jaws which have been separated from the heads of the embryos can be seen in Fig. 3D (10,5-day embryo) and Fig. 4D (12,5-day embryo). Each lower jaw was placed in a glass cavity block in 0,04% collagenase in Tyrode's solution and incubated at 21°C for 20 – 40 minutes.

After the period of incubation in collagenase, both the forelimbs and lower jaws were rinsed in Tyrode's solution and the epithelium and mesenchyme of each piece was manually separated from each other. The forelimb bud epithelium was separated from its own mesenchyme by gentle pipetting of the buds through a thin glass pipette. The lower

jaws were firmly pinned on to a resin-filled cavity block with Tyrode's solution and the epithelium and mesenchyme were manually separated using an ophthalmic knife and fine needle, with the visual aid of a transmitted light stereomicroscope. The 12,5-day embryonic lower jaws were cut medially into halves.

A modified Trowell culturing system (Trowell, 1954) was used for culturing explants *in vitro* in order to adhere as closely as possible to the techniques used by Ohazama *et al.* (2004). Stainless steel rafts were heat sterilised and placed in 4-well Nunc dishes (Nunclon, Roskilde, Denmark). Approximately 700µl of fresh tissue-culture medium was pipetted into each of the wells. The amount of medium used depended on the varying heights of the stainless steel rafts when placed in each well. Nucleopore membrane filters (0,05µm pore diameter, Millipore Corporation, Ireland) were sterilised by immersing them in alcohol (95%, 70%, 2X sterile distilled H_2O). The membrane filters were then placed in sterile serum-free DMEM supplemented with glutamax and 0,1% pen/strep (Ref #: 41966-029, Gibco, Invitrogen) for 30 minutes prior to placing them on the rafts. One filter was then placed on each of the rafts and any air bubbles that may have formed below the membrane filters were removed.

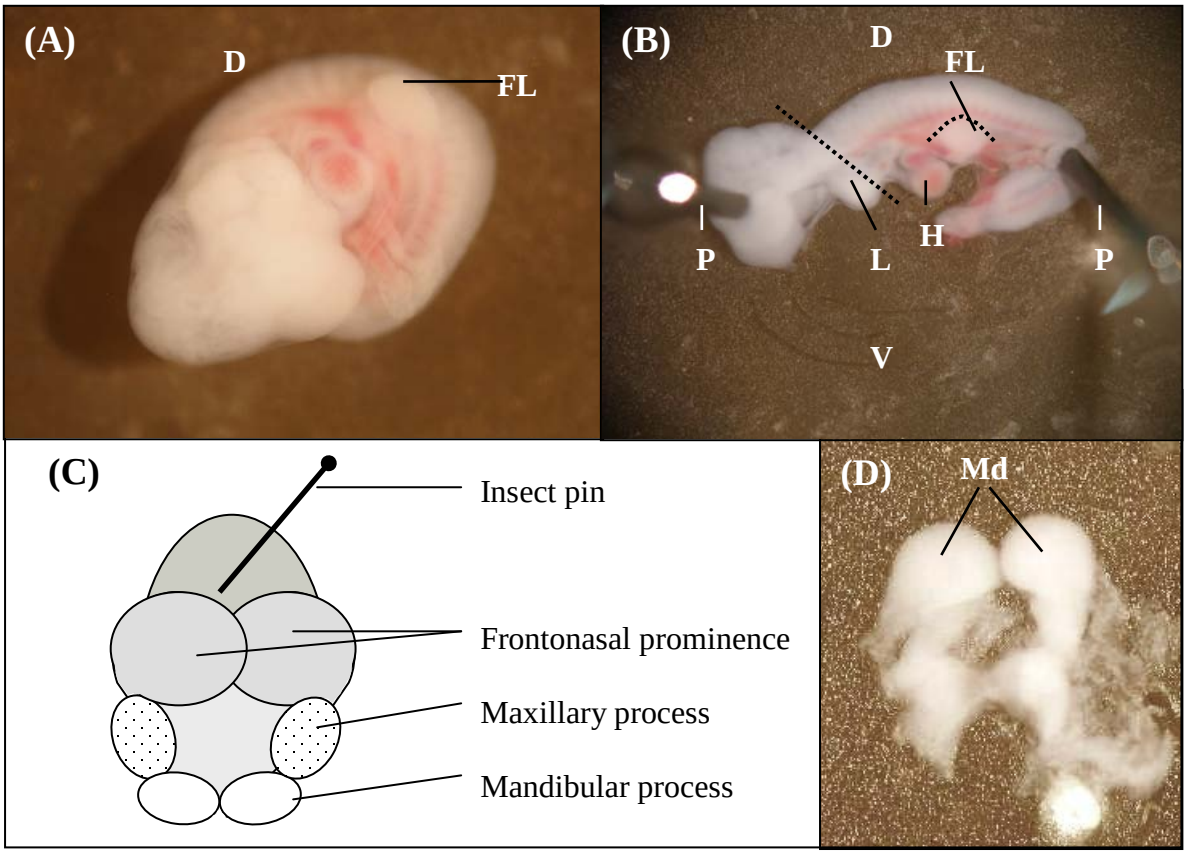

Figure 3: Photos showing the removal of the lower jaw and forelimbs from a 10,5 day mouse embryo. (A) The embryo was removed from the uterine horn and placed in a black wax dish (x 12,5). (B) The embryo was pinned on its side to facilitate removal of the head and forelimbs (incisions indicated by interrupted lines) (x 10,5). (C) The head was pinned on to its dorsal surface. The diagram shows the ventral surface of the head with the maxillary and mandibular processes. (D) A transverse view of the lower jaw after excision showing the two mandibular processes that are not yet fused (x 25). D- dorsal surface, V- ventral surface, FL- forelimb, L- lower jaw, P- insect pin, H- heart, Mx- maxillary process, Md- mandibular process. (All the embryos were dissected and photographed with the aid of a stereomicroscope).

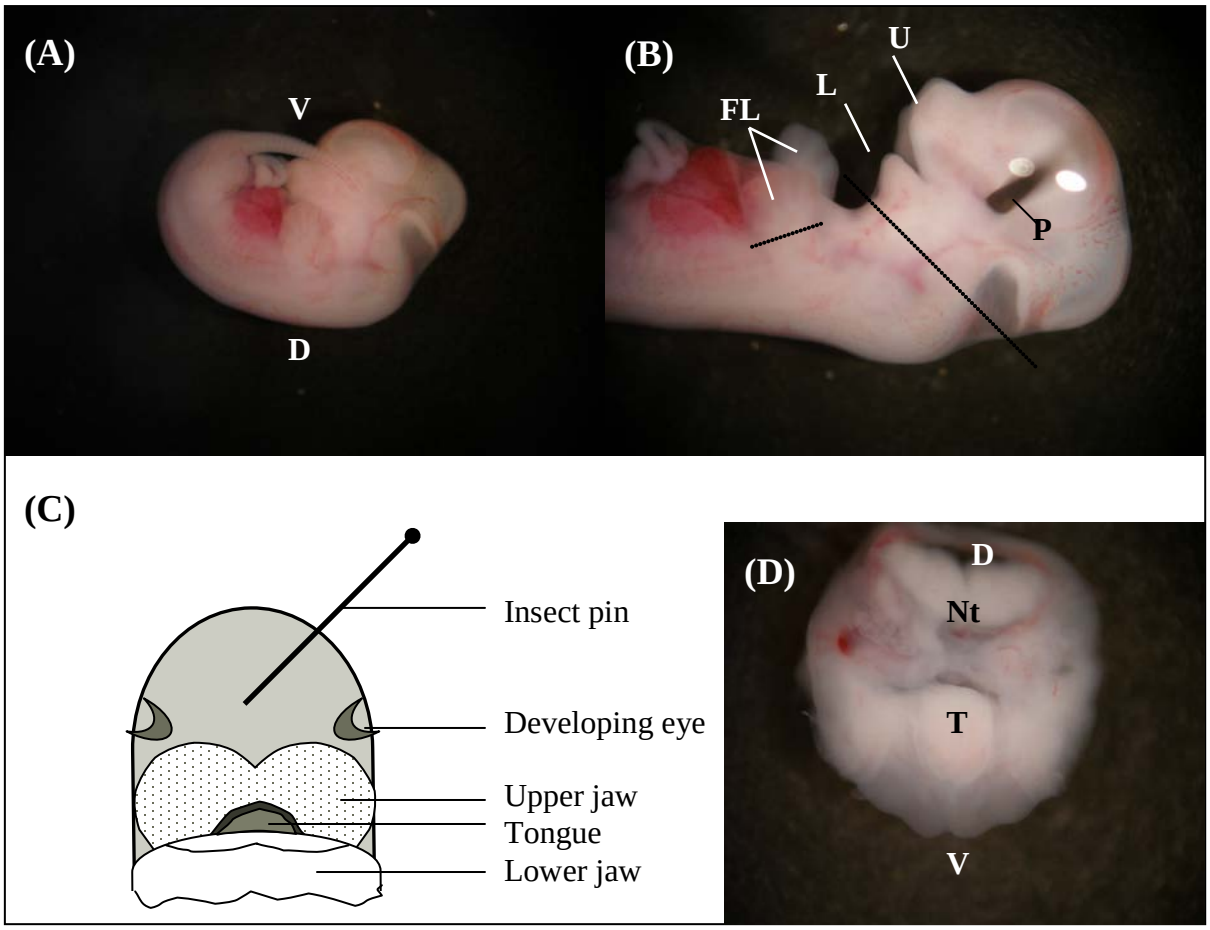

Figure 4: Photos showing the removal of the lower jaw and forelimbs from a 12,5 day mouse embryo. (A) The embryo was removed from the uterine horn and placed in a black wax dish (x 7). (B) The embryo was pinned on its side to facilitate removal of the head and forelimbs (incisions indicated by interrupted lines) (x 12,5). (C) The head was pinned on to its dorsal surface. The diagram shows the ventral surface of the head with the upper and lower jaws clearly exposed. (D) Transverse view of the lower jaw together with the tongue and neural tube after excision (x 25). V- ventral surface, D- dorsal surface, FL- forelimb, L- lower jaw, U- upper jaw, P- insect pin, T- tongue, Nt- neural tube. (All the embryos were dissected and photographed with the aid of a stereomicroscope).

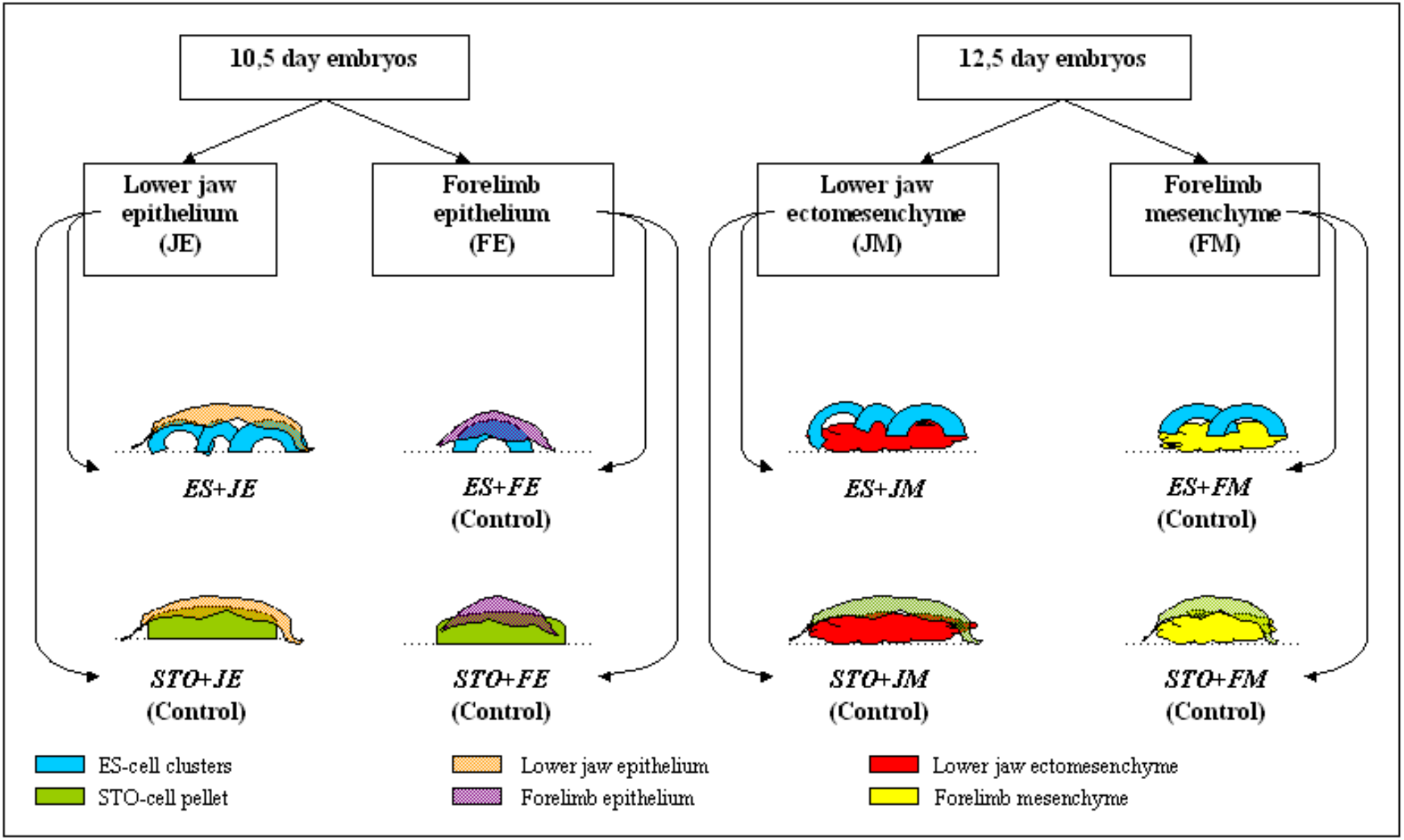

Figure 5: Diagram showing the different combinations of mouse embryonic lower jaw epithelium or ectomesenchyme and forelimb epithelium or mesenchyme with ES-cell clusters or STO-cell pellets.

The oral epithelium of the 10,5-day embryonic lower jaws (N=54) as well as the epithelium of the 10,5-day embryonic forelimb buds (N=31) were combined with ES cell clusters (Fig. 5), or with STO cell pellets (as controls) (Fig. 5). Clusters of ES cells (approximately three to five) or a pellet of STO cells was placed on each of the prepared nucleopore membrane filters. As the lower jaw is much larger than the forelimb, the number of ES cell clusters and STO pellets used in each combination were selected to replace volume for volume the ectomesenchyme which had been separated from the epithelium. The oral epithelium of each lower jaw was first flattened out on a resin-filled cavity block and then placed on top of either a group of ES cell clusters (N=31) or a STO cell pellet (N=23). The forelimb surface-epithelium was similarly placed on a group of ES cell clusters (N=14) to serve as a control for the oral epithelial explants, or on a STO pellet (N=17), as a control for the ES cell containing cultures.

The ectomesenchyme of the 12,5-day embryonic lower jaw halves (N=79) and the mesenchyme of the forelimbs (N=35) of the same age were also respectively combined with ES cell clusters (Fig. 5), or with STO cell pellets (as controls) (Fig. 5). In these experiments the forelimb mesenchyme or ectomesenchyme of the lower jaw was first placed on the prepared nucleopore membrane filter. Clusters of ES cells (approximately three to five, to substitute area for area the epithelium that had been removed) were placed on each of the dissected ectomesenchyme (N=48) and forelimb mesenchyme (N=17). The STO cell pellets used as a control for the ES cells were similarly placed on separate oral ectomesenchyme (N=31) or forelimb mesenchymal tissue (N=18).

All the explants were cultured at the air-medium interface for three days at 37 C in a humidified incubator containing 5% CO_2 in air. The medium of the explants was changed

every second day. After three days of culturing the explants were retrieved. Some control and experimental explants were fixed and processed for histological and immunocytochemical analysis as described later (For the numbers of explants, see Results, Table 1 and 2). The remainder of the explants were used as grafts for either chorio-allantoic membrane (CAM) grafting or renal subcapsular grafting experiments (For numbers of grafts, see Results, Table 1 and 2).

2.7 Chorio-allantoic membrane grafting

Chorio-allantoic membrane grafting is an established technique, which provides a nutritive and sterile environment and allows the prolongation of *in vitro*- cultured grafts (von Studitsky, 1939; Glasstone, 1954). Combinations of both control and experimental groups were grafted on to the chorio-allantoic membrane of fertile chicken eggs in order to allow for further development. Fertile chicken eggs (*Gallus gallus*, Rhode Island Red and New Hampshire) were obtained from the Central Animal Unit at the University of the Witwatersrand and incubated in a humidified incubator at 37C. The host chick embryos were prepared on the third day of incubation. Each egg was placed on its side in a dish lined with cotton wool. A hole was made into the air sac at the blunt tip of the egg to allow air to escape. A syringe and thick needle was used to extract 1,5ml of albumin from the pointed end of the egg. A window was then cut into the side of the shell of each fertilised egg and the shell membrane removed. A drop of chick Ringer containing 0,01% pen/strep (Appendix G) was placed on the chorio-allantoic membrane to prevent it from drying out. The 0,01% pen/strep was added to the chick Ringer to prevent any infection. Each window was sealed with tape and the eggs were incubated for an additional six days in a humidified incubator at 37˚C.

The tape was removed from the window of each host egg on the tenth day of incubation. Small branching blood vessels were scored using a fine needle. Care was taken not to cause excessive damage to the blood vessels, which could lead to increased bleeding. Each graft was transplanted on to the chorio-allantoic membrane in the vicinity of the scored membrane (including blood vessels) using a fine pipette or small spatula. Excess fluid was removed using a pipette to prevent the graft from floating away from the transplantation site. The window was then sealed with tape and the host egg was incubated for seven days before retrieval of the graft on the seventeenth day of host incubation.

2.8 Renal subcapsular grafting

Renal subcapsular grafting offers a more suitable environment for long-term culturing than CAM grafting, by preventing the dehydration of tissue. This method of prolonging the culturing of tooth primordia has been used with great success (Bei *et al*., 2000; Ohazama *et al*., 2004; Cai *et al*., 2007; Komine *et al*., 2007; Nakao *et al.,* 2007) The information for this technique was obtained from Brody *et al.* (1998) and adapted for this study. All surgical procedures were performed under aseptic conditions, under sterile theatre conditions at the University of the Witwatersrand Central Animal Unit, with the assistance of the Central Animal Services (C.A.S.) veterinarians and nurse.

2.8.1 Preparation of the hosts

Twelve NMRI X NMRI male mice were weighed and anaesthetized accordingly by a C.A.S. veterinary nurse using a 4:1 solution of Anaket-V and 2% Chanazine (0,1ml/100g). The skin on the back of each mouse was shaved and cleaned with

disinfectant. The shaved area of skin was approximately 5cm X 4cm overlying the implantation site. Each mouse was placed on a sterile drape over a hot pad and given oxygen with 2% Isofor inhalation anaesthetic during the procedure. A 3cm longitudinal incision was made in the skin about 1cm above the tail. A smaller incision was made in the posterior abdominal wall at the site of each kidney through which the kidney was exteriorised by gently pressing on either side of the kidney with the fingertips.

2.8.2 Insertion of the grafts

The grafts were inserted with the aid of a stereomicroscope. Each kidney was moistened with sterile gauze dipped in sterile PBS to avoid dehydration and tearing of the kidney capsule. The capsule was lifted using a fine forceps and pierced using a #11 scalpel blade. A small glass-ball pipette was inserted under the capsule to loosen it from the underlying parenchyma, forming a pocket for the graft.

The grafts that had been prepared three days prior to implantation were placed into sterile cavity dishes with fresh tissue-culture medium. Each host received one or two grafts consisting of ES cells combined with either oral ectomesenchyme or oral epithelium from the lower jaw. These were grafted under the left kidney capsule. The grafts implanted under the right kidney capsule consisted of either an intact lower jaw to serve as control for the grafting technique, or STO cells combined with either oral ectomesenchyme or oral epithelium from the lower jaw. The latter served as a measure to determine whether the ES cells alone were the determining factor in tooth rudiment formation. Each graft was picked up from the cavity dish using a small spatula and placed on to the kidney next to the implantation site. The capsule was then lifted using fine forceps and the graft was carefully pushed into the pocket using the glass-ball

pipette. The kidney was carefully pushed back into the abdomen. The abdominal wall was sutured using 4-0 vicryl sutures and the incision in the skin was sutured using 4-0 nylon sutures. The mouse was then revived using Antisedan (0,04 ml/kg) and allowed to recover from the anaesthetic before returning it to the living quarters. The mouse was also given 10% Temgesic for pain (0,05mg/kg).

The mice that served as hosts for the renal subcapsular grafting were carefully monitored in the three weeks following grafting to detect infection or illness due to the surgical procedure. Twenty-one days after implantation of the grafts, each mouse was euthanased using CO_2. One of the mice had to be euthanased nineteen days after implantation of the grafts due to an untreatable eczema it had developed on the back of its neck after the surgery. The kidneys containing the developed grafts were removed and placed in 4% paraformaldehyde for processing (For the numbers of subcapsular grafts, see Table 1 and 2).

2.9 Processing of the tissue

All the three-day *in vitro* cultured explants, as well as the grafts that were retrieved from host eggs were fixed in 4% paraformaldehyde (Appendix H) for two hours. The kidneys obtained from the renal subcapsular grafting were fixed for eight hours. The explants that were cultured for three days *in vitro,* were noticeably smaller than the CAM-grafts and the renal subcapsular (RS) grafts. The former explants were often difficult to see after they were processed and were therefore placed in eosin diluted with 50% alcohol for one minute in order to view them in the paraffin wax. The CAM and RS grafts, which showed more advanced development than the explants were placed in formic citric acid for two to seven hours in order to decalcify any mineralised tissue that may have been present

(Appendix I). The decalcification process was monitored by adding a few drops of ammonium oxalate to the formic citric acid and checking for a cloudy discolouration of the chemical (Appendix J).

The three-day *in vitro* cultured explants and the CAM grafts were manually dehydrated through a graded series of alcohols (50%, 70%, 95%, 100%), cleared in two changes of xylene and embedded in paraffin wax. The kidneys containing the grafts obtained from the RS grafting were placed in plastic baskets in an automatic processor and immersed in the following solutions; 70% alcohol for one hour, 3x 95% alcohol (five hours in total), 3x absolute alcohol (four hours in total), 2x chloroform (three hours in total). The grafts were infiltrated with two changes of paraffin wax and then embedded in paraffin wax.

2.10 Histological analysis of the sectioned explants and grafts

For histological analysis the blocks containing the tissue were serially sectioned at 4µm using a sledge microtome (Leica, Germany, Model 1400). The sections were floated on to distilled water in a water bath and collected on glass slides coated with 2% 3-aminopropyl-triethoxysilane (Sigma, USA, Lot # 023K0642) (Appendix K).

For morphological analysis with haematoxylin and eosin, serial sections of 12 – 16 µm apart were dewaxed by dipping them in xylene twice for five minutes each. Sections were then hydrated through a graded series of alcohols (100%, 100%, 95%) to water. The sections were dipped in Mayer's haematoxylin for five minutes and blued under running tap water. Sections were then dipped in eosin for thirty seconds, rinsed in tap water, dehydrated through a graded series of alcohols (95%, 100%, 100%), cleared in xylene and mounted in Entellan.

Sections of all the explants and CAM and RS grafts were specifically selected to correlate with the H&E stained sections. These were stained with a Periodic acid Shiff's stain (McManus, 1946) in order to histologically identify PAS-positive structures such as basement membranes and glycogen. The use of commercial diastase to digest glycogen is a more standardized method in the PAS staining technique, as opposed to the use of saliva, which contains salivary amylases (Bancroft and Gamble, 2002). However, a trial run using saliva from a variety of donors to digest glycogen yielded identical results for the PAS-stained sections. Salivary amylase was therefore chosen as the preferred glycogen digestion enzyme. Two serial sections of each explant and graft were hydrated through a graded series of alcohols (100%, 100%, 95%) to water. One section of each set was incubated with saliva in a closed petri dish at 37°C for fifteen minutes, while the other section was left in water. Both sections were then rinsed in tap water. All the sections were treated with 1% periodic acid (Appendix L) for five minutes and then rinsed in distilled water. The sections were incubated in Shiff's Reagent (Appendix M) for fifteen minutes at room temperature, after which they were washed well in distilled water to remove excess Schiff's Reagent. The sections were briefly counterstained in Mayer's haematoxylin for one minute, rinsed in tap water, dehydrated through a graded series of alcohols (95%, 100%, 100%), cleared in xylene and mounted in Entellan.

2.11 Immunolocalisation of Oct-4 in undifferentiated ES cells

Oct-4 is a protein, which is expressed in undifferentiated embryonic and adult stem cells, as well as in certain carcinoma cells (Pesce and Scholer, 2001; Gidekel *et al*., 2003; Matthai *et al*., 2006). Sections of all the explants and grafts containing combinations of embryonic tissue and ES cells were labelled for the presence of Oct-4 using an indirect

immunolocalisation technique. To establish whether the technique was specific enough for Oct-4 labelling, sections of explants and grafts containing embryonic tissue in combination with STO cells were also used, as the STO cells were not expected to express Oct-4. Sections were dewaxed in two changes of xylene and rehydrated through a graded series of alcohols to water. The sections were then washed in distilled water twice for five minutes each. Antigen retrieval was performed by boiling the sections in sodium citrate (Appendix N) for ten minutes. The sections were then allowed to cool for thirty minutes. Exogenous peroxidase was blocked by incubating sections in 3% hydrogen peroxide for thirty minutes. Non-specific labelling was blocked by covering the sections with a blocking solution of 5% swine serum in phosphate-buffered saline (PBS) for one hour at room temperature. The primary anti-serum, polyclonal anti-Oct-4 antibody (1:200) (Biovision, USA, Cat #. 3576-100), diluted in the blocking solution, was applied and sections were incubated overnight at 4°C.

The sections were washed in PBS three times for five minutes each, prior to incubation with the secondary antiserum. Peroxidase-conjugated polyclonal swine anti-rabbit antiserum (1:100) (Dako Cytomation, Denmark, Cat #. P0217) was applied to the anti-Oct-4 immunolocalized sections for thirty minutes at room temperature. Sections were washed in PBS for fifteen minutes before the site of the antigen was revealed with a diaminobenzidine (DAB) solution containing 0,015% hydrogen peroxide (Appendix O). The sections were dehydrated through a graded series of alcohols, cleared in xylene and mounted in Entellan.

Negative controls for the immunolocalisation technique were carried out on adjacent serial sections by replacing the primary antibody or the secondary antibody respectively

with PBS alone or 5% normal swine serum in PBS. An additional control included the labelling of sections of the lower jaw to determine whether the embryonic tissue used in this study expressed Oct-4. Sections of ES cell pellets were included with each immunolocalisation, to determine whether the procedure had worked, as the undifferentiated ES cells in the pellets were expected to express Oct-4.

2.12 Immunolocalisation of Msx1 in the mesenchymal tissue of all the explants and grafts

Msx1 is a mesenchymal marker expressed in tissue where there is an interaction between the epithelium and underlying mesenchyme (Mitsiadis *et al.*, 2003; Aberg *et al.*, 2004; Ohazama *et al.*, 2004; Kriangkrai *et al.*, 2006). Sections of the explants and grafts containing ES cells in combination with oral epithelium or ectomesenchyme from the lower jaw were immunolabelled for Msx1 as a marker for the presence of tooth formation. Sections of explants and grafts containing ES cells in combination with forelimb epithelium or mesenchyme were also immunolabelled to see if the mesenchyme or ES cells were induced to express Msx1. As with Oct-4 labelling, the sections were first dewaxed in two changes of xylene and rehydrated through a graded series of alcohols to water. Exogenous peroxidase was blocked by incubating sections in 3% hydrogen peroxide in distilled water for twenty minutes. Sections were then rinsed in distilled water after which they were quenched for thirty minutes using 50mM ammonium chloride. Cellular membranes were permeabilised for seven minutes using proteinase K (Appendix P), after which the reaction was stopped by washing slides in PBS. Non-specific labelling was blocked by covering the sections with 10% normal swine serum in PBS. The optimal dilution of antibody was first determined by the researcher. The primary anti-serum, polyclonal anti-Msx1 antibody (1:1000) (United States Biological, Massachusetts,

USA, Cat #. M4692-74), diluted in 10% normal swine serum in PBS, was applied and sections were incubated overnight at 4°C.

To prevent non-specific binding of the secondary antibody, the sections were washed in PBS containing 1% horse serum for fifteen minutes before incubation with secondary antiserum. Biotin-conjugated goat anti-rabbit antiserum (1:1000) (Molecular Probes, USA, Cat #. B-2770) was applied to the anti-Msx1 immunolocalized sections and the sections were incubated for ninety minutes at 37°C. Sections were washed in PBS for fifteen minutes and treated with an ABC reagent kit prepared according to the manufacturer's specifications (Vector Laboratories, CA, Cat #. PK-6200) for thirty minutes. Sections were again washed in three changes of PBS, before the site of the antigen was revealed with DAB containing 0,03% hydrogen peroxide. The sections were dehydrated through a graded series of alcohols, cleared in xylene and mounted in Entellan.

Negative controls for the immunolocalisation technique were carried out on adjacent serial sections by replacing the primary antibody or the secondary antibody respectively with 10% normal swine serum or PBS. Sections of lower jaw, known to contain developing teeth, from E13,5 and E15,5 mouse embryos, as well as sections of forelimb taken from these embryos, were included in each immunolocalised batch, to serve as a positive control.

3. RESULTS

3.1 Terminology

For convenience, the different groups of explants and grafts will be referred to as follows: Explants and grafts consisting of ES cells in combination with 10,5-day embryonic oral **epithelium** of the lower **jaw** will be referred to as ***ES+JE***; Explants and grafts consisting of ES cells in combination with 10,5-day embryonic **forelimb** surface-**epithelium** will be referred to as ***ES+FE***; Explants and grafts consisting of ES cells in combination with 12,5-day embryonic oral **ectomesenchyme** of the lower **jaw** will be referred to as ***ES+JM***; Explants and grafts consisting of ES cells in combination with 12,5-day embryonic **forelimb mesenchyme** will be referred to as ***ES+FM***.

Explants and grafts consisting of STO cells in combination with 10,5-day embryonic oral **epithelium** of the lower **jaw** will be referred to as ***STO+JE***; Explants and grafts consisting of STO cells in combination with 10,5-day embryonic **forelimb** surface-**epithelium** will be referred to as ***STO+FE***; Explants and grafts consisting of STO cells in combination with 12,5-day embryonic oral **ectomesenchyme** of the lower **jaw** will be referred to as ***STO+JM***; Explants and grafts consisting of STO cells in combination with 12,5-day embryonic **forelimb mesenchyme** will be referred to as ***STO+FM***.

In this study, the lower jaws that were removed from the mouse embryos and cultured for three days *in vitro* will be referred to as **cultured lower jaws**. The lower jaws that had been removed from mouse embryos at equivalent ages to the three-day cultured jaws will be referred to as **intact jaws**.

3.2 Animal usage

Sixteen NMRI X NMRI pregnant female mice were used in this study. Seven of these were sacrificed on 10,5 days of pregnancy, of which a total of seventy-seven embryos were obtained. Nine females were sacrificed on 12,5 days of pregnancy. This yielded a total of seventy-three embryos. An average of nine embryos per pregnant female was obtained.

3.3 The number of embryos used versus the total yield of explants and grafts for each group

3.3.1 Explants and grafts of 10,5-day embryos

Explants of 10,5-day lower jaws, *STO+JE* or *ES+JE* were cultured for three days *in vitro*, followed by either fixation or by CAM grafting for seven days or as RS grafts for twenty-one days (Table 1). It is of interest that none of the *STO+JE* CAM grafts or *STO+JE* RS grafts survived.

The numbers of explants of forelimbs, *STO+FE* and *ES+FE* that were cultured for three days *in vitro*, followed by fixation or CAM grafting for seven days are shown in Table 1. Only one of the *STO+FE* and one of the *ES+FE* CAM grafts survived (Table 1).

3.3.2 Explants and grafts of 12,5-day embryos

The lower jaw of seventy 12,5-day embryos was dissected and cultured alone, or as *STO+JM* or *ES+JM* for three days *in vitro*, followed by fixation or CAM grafting for seven days or as RS grafts for twenty-one days, the numbers of which are shown in Table 2. Only one of the *STO+JM* CAM grafts survived.

Explants of forelimbs, or *STO+FM* and *ES+FM* were cultured for three days *in vitro, after* which some of the explants were used for CAM grafting. The number of explants and grafts cultured and retrieved from these procedures is shown in Table 2.

3.4 Comparison of the appearance of the different explants in culture

Nearly all of the 10,5-day cultured lower jaw (Fig. 6E) and whole forelimb (Fig. 6G) explants appeared to have spread out over the nucleopore membrane and increased only marginally in size. Both 12,5-day cultured lower jaw (Fig. 6F) and forelimb (Fig. 6H) explants appeared to have increased in size during the *in vitro* culture period.

The 10,5-day *ES+JE* explants (Fig. 6A) appeared slightly translucent. The folding of the epithelium could be seen in culture and the explants showed some internal detail. The 10,5-day *STO+JE* explants (Fig. 6B) appeared as an opaque mass with no internal detail or evidence of the presence of epithelium. Histological analysis of these explants showed that they survived the culturing period.

The 12,5-day *ES+JM* explants (Fig. 6C) as well as the *STO+JM* explants (Fig. 6D) appeared to have increased in size during the three-day *in vitro*-culture period. Both groups of explants also showed an increase in blood vessels (Fig. 6C,D). By the third day of culturing the STO cell pellets which were combined with the 12,5-day embryonic lower jaw ectomesenchyme had spread across the tissue, obscuring most of the internal detail of the tissue.

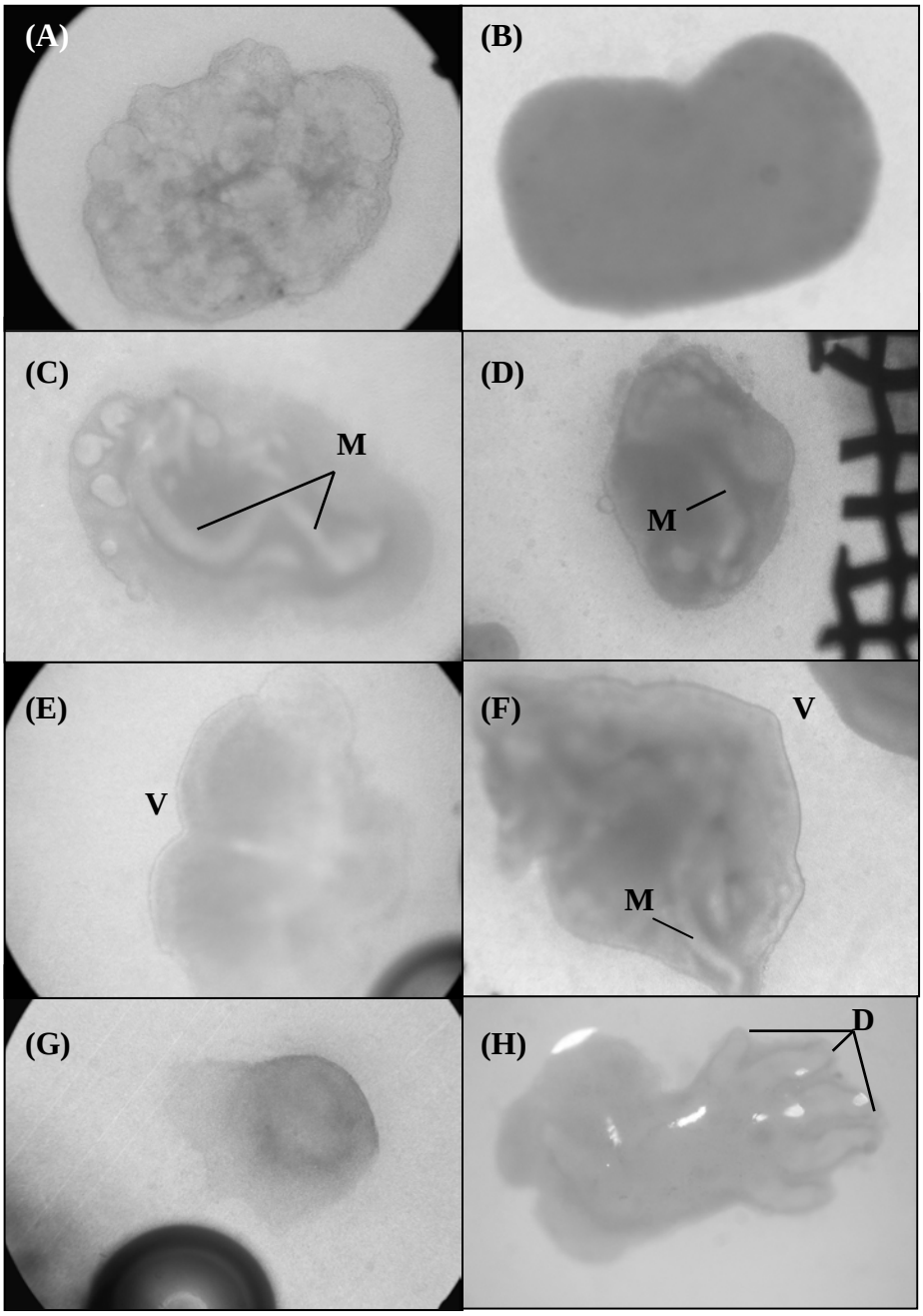

Figure 6: Explants of 10,5-day and 12,5-day embryonic lower jaw and forelimb tissue cultured alone or in combination with ES cells or STO cells for three days *in vitro*. (A) A *ES+JE* explant. (B) A *STO+JE* explant. (C) A *ES+JM* explant. (D) A *STO+JM* explant. (E) A cultured 10,5-day embryonic lower jaw, (F) A cultured 12,5-day embryonic lower jaw. (G) A cultured 10,5-day embryonic forelimb showing minimal development, (H) A cultured 12,5-day embryonic forelimb. M- Meckel's cartilage, V- ventral surface of the jaw, D- digits. (x 40) (Phase contrast microscopy)

3.5 Histological analysis of all the explants and grafts retrieved after the specified culture periods

3.5.1 Histological sections of three-day cultured lower jaws of 10,5-day and 12,5-day embryos, compared with intact lower jaws of equivalent ages.

The three-day cultured lower jaws of 10,5-day embryos (Fig. 7A) appeared flattened with some superficial cuboidal and columnar epithelium visible (Fig. 7B). The deeper layers of these explants consisted of mesenchymal cells, but no Meckel's cartilage or blood vessels were visible. No tooth formation was observed in the cultured 10,5-day lower jaws. In comparison, the 13,5-day intact lower jaws showed an extensive superficial layer of epithelium lined by a distinct basement membrane. The internal ectomesenchyme contained a few blood vessels as well as Meckel's cartilage. Tooth formation was identified by the tooth bud-like structures that were present on either side of a developing tongue (Fig. 7C,D).

The three-day cultured lower jaws of 12,5-day embryos appeared different from intact lower jaws of embryos of an equivalent embryonic age (15,5 days) (Fig. 8C,D). One of the ten cultured 12,5-day lower jaws (Fig. 8A,B) was similar in appearance to the 13,5-day intact lower jaws (Fig. 7C,D). This cultured 12,5-day lower jaw showed some superficial epithelium lined by a distinct basement membrane and contained Meckel's cartilage and a few blood vessels. Two developing tooth primordia were also visible (Fig. 8A,B). The other nine cultured 12,5-day lower jaws, however were slightly flattened with

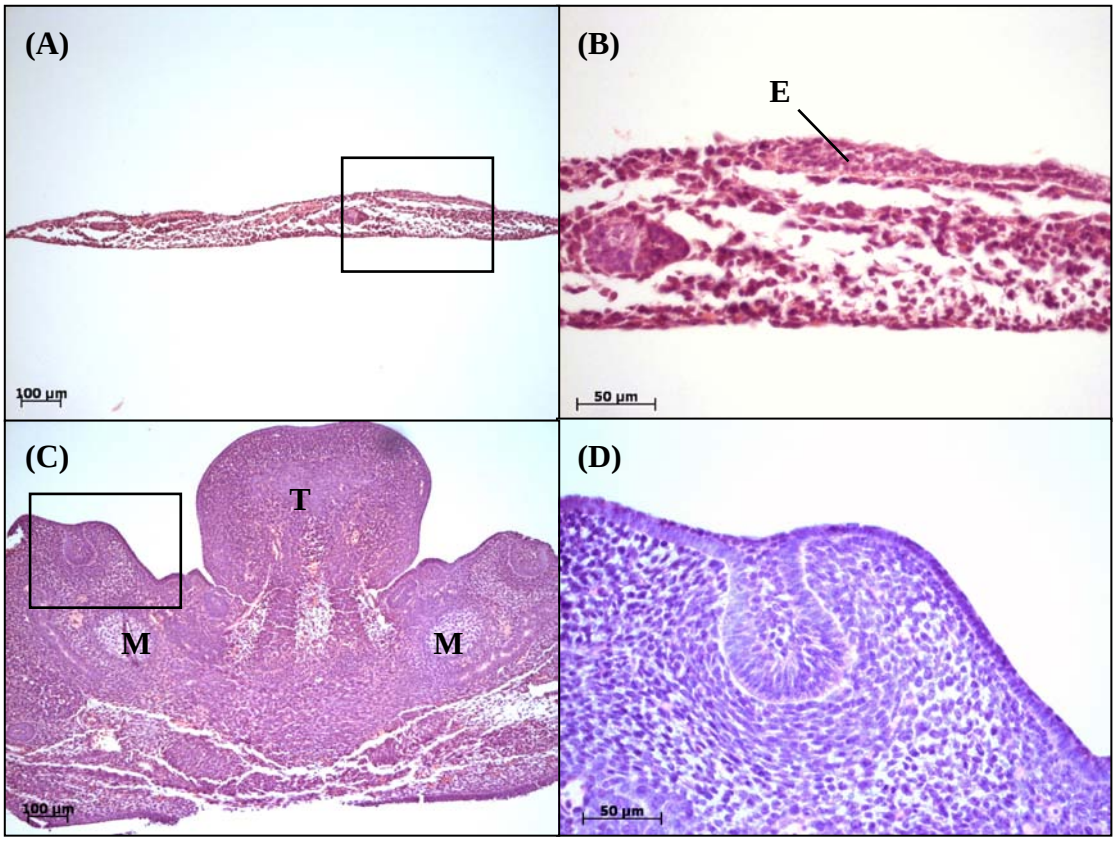

Figure 7: (A) An explant of 10,5-day embryonic lower jaw cultured for three days *in vitro*. The explant is flattened with no morphological signs of tooth development (x100). (B) An enlarged view of the marked area in (A) showing some cuboidal epithelium on the superficial surface of the explant (x400). (C) A 13,5 day intact lower jaw (x100). (D) An enlarged view of the marked area in (C) showing a developing tooth bud (x400). E- epithelium; T- tongue; M- Meckel's cartilage. (H&E, Bright field microscopy)

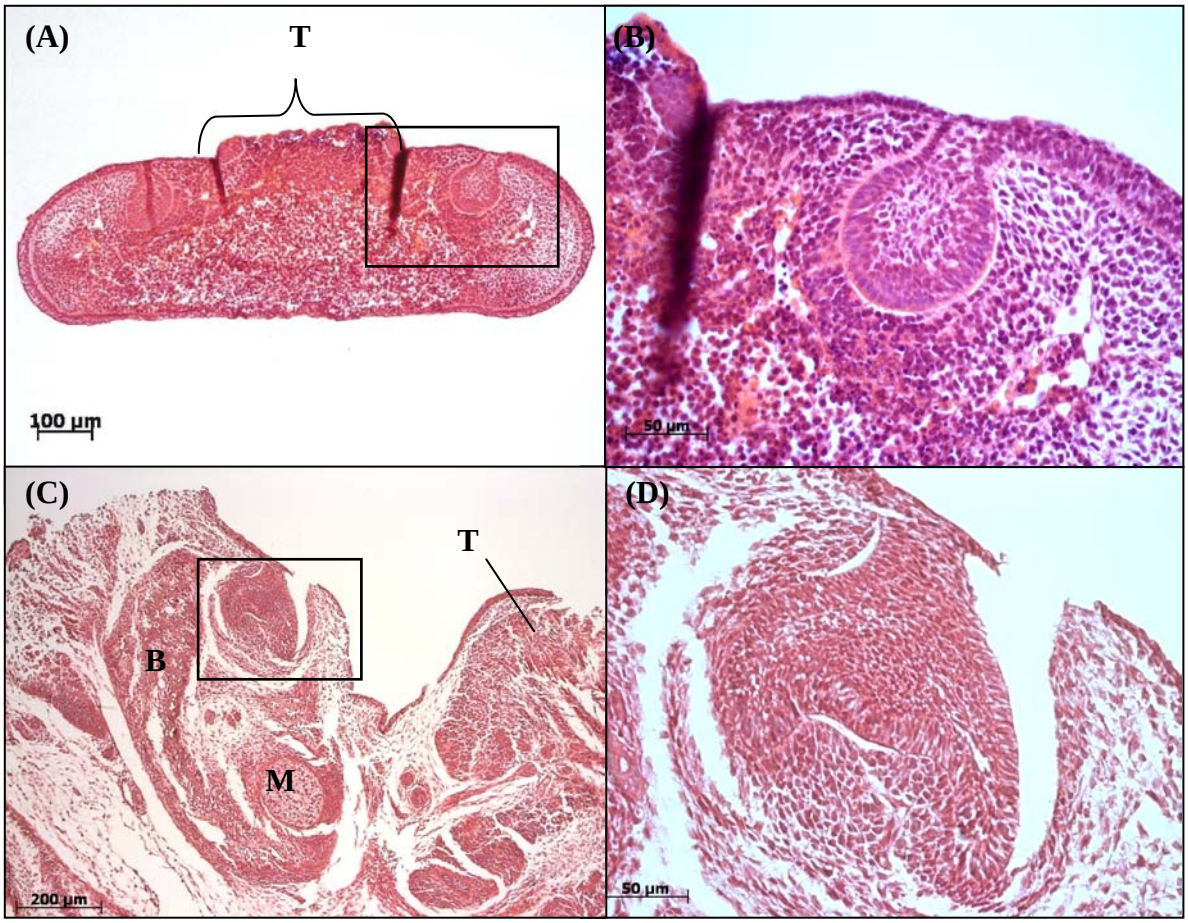

Figure 8: (A) An explant of 12,5-day embryonic lower jaw cultured for three days *in vitro* (x100). (B) An enlarged view of the marked area in (A) showing a developing tooth bud (x400). (C) A 15,5 day intact lower jaw (x100). (D) An enlarged view of the marked area in (C) showing a developing tooth (x400). T- tongue; B- Bone; M- Meckel's cartilage. (H&E, Bright field microscopy)

minimal superficial cuboidal and columnar epithelium visible. Only two of these nine explants showed some delayed tooth formation. The deeper layers of these cultured 12,5-day explants contained ectomesenchyme, Meckel's cartilage and few blood vessels. In comparison, the 15,5-day intact lower jaws showed an extensive superficial layer of epithelium lined by a distinct basement membrane. The internal ectomesenchyme contained some blood vessels as well as Meckel's cartilage. Tooth formation was observed as shown by the cap-staged tooth primordia that were present on either side of the tongue (Fig. 8D).

3.5.2 Histological comparison of explants of *ES+JE*, *STO+JE, ES+FE* and *STO+FE* cultured for three days *in vitro*.

In comparing the groups of *ES+JE* (N=7) and *STO+JE* (N=7) explants, clusters of what appeared to be ES cells were observed to be present in the deeper layers of the *ES+JE* explants (N=6) (Fig. 9A,B), while the *STO+JE* explants did not contain any ES-like cell clusters (Fig. 10A). The ES-like cells were identified by their rounded nuclei, encapsulated by a pale eosinophilic extracellular matrix, overlaid by the oral epithelium in the majority of the *ES+JE* explants (Fig. 9B). This extracellular matrix also stained positive with the PAS stain (Fig. 9C). The STO cells in six of the *STO+JE* explants were identified as elongated cells with a characteristic fibroblast appearance. No epithelium was evident in the *STO+JE* explants although the cell shape varied throughout the explants (Fig. 10B). Squamous cells were observed lining three of the *STO+JE* explants (Fig. 10C). In contrast, the majority of the *ES+JE* explants contained tissue consisting of visibly organised rows of cuboidal to columnar cells, which resembled epithelium, both in the superficial and deep layers of these explants (N=6) (Fig. 9A,B,D). Neither the *ES+JE* nor *STO+JE* explants contained Meckel's cartilage or blood vessels. Small rounded

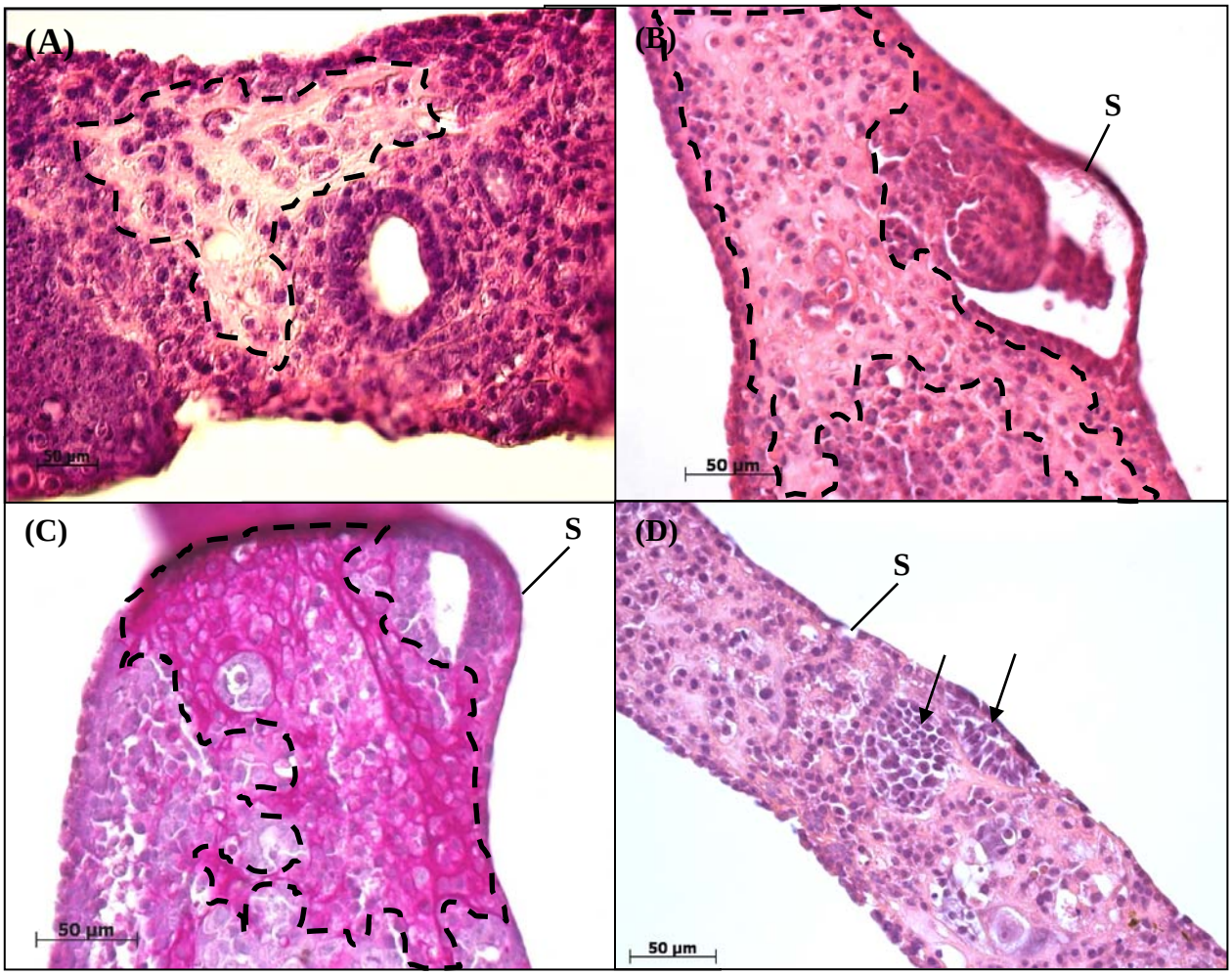

Figure 9: Explants of *ES+JE*, cultured for three days *in vitro*. (A) A cluster of ES cells can be seen (interrupted lines) consisting of rounded cells surrounded by a pale eosinophilic extracellular matrix (x400). (B) Cuboidal and columnar epithelium is present in the superficial layer of the explant, overlying what appears to be a large ES cell cluster (outlined) (x400). (C) A section adjacent to (B) that shows a large area of PAS-positive staining (outlined) that correlates to that of the ES cluster in (B) (x400). (D) What appears to be some columnar epithelium can be seen in the superficial and deeper layers of the explant (x400). S- Superficial surface. (H&E (A,B and D), PAS (C), Bright field microscopy)

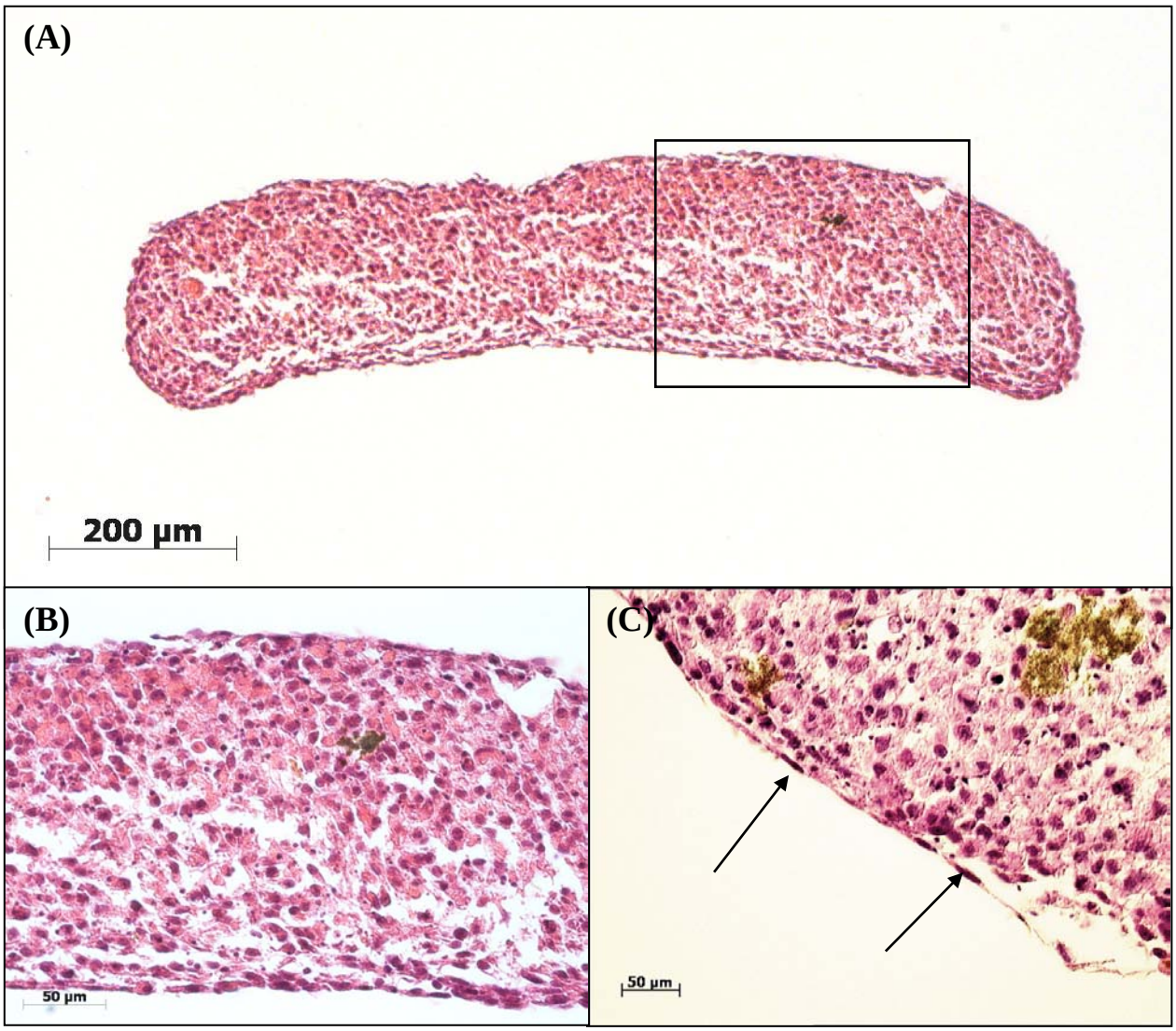

Figure 10: (A) An explant of *STO+JE*, cultured for three days *in vitro* (x100). The surface epithelium is not clearly defined as the epithelium combined with ES cells (Fig. 9). (B) An enlarged area of (A) showing the variation in cell shape (x400). (C) An explant consisting of 10,5-day embryonic oral epithelium of the lower jaw combined with a STO cell pellet, cultured for three days *in vitro,* showing the single layer of squamous cells (arrows) that line some of these explants (x400). (H&E, Bright field microscopy)

intensely basophilic nuclei were observed in some of the *ES+JE* (N=4) and *STO+JE* (N=7) explants.

Explants of *ES+FE* (N=7) and *STO+FE* (N=9) served as negative control groups for *ES+JE* and *STO+JE* explants respectively. In six of the *ES+FE* explants, the presence of what appeared to be cuboidal and columnar epithelium in the superficial layers, overlying the ES cell clusters was observed (Fig. 11A,C). However, none of the *STO+FE* explants showed any clearly defined epithelial cells or cell layers (Fig. 12). The cells in the deep layers of the *STO+FE* explants varied in shape. Some of the cells were fibroblastic in appearance and more loosely arranged, while other cells appeared more rounded (Fig. 12B). Squamous cells were observed lining four of the *STO+FE* explants, as was seen in the *STO+JE* explants. Neither *ES+FE* nor *STO+FE* explants contained any cartilage. Small rounded intensely basophilic nuclei were observed in many of the *ES+FE* and *STO+FE* explants.

In both the *ES+JE* and *ES+FE* explants, it was sometimes difficult to determine whether groups of highly concentrated cells were epithelial or mesenchymal in origin, as a distinct basement membrane was not always seen. Five of the *ES+JE* explants and five of the *ES+FE* explants however showed the presence of what seemed to be intact basement membranes, which stained PAS-positive. However, these could also have been part of the ES cell clusters (Fig. 11B,D).

None of the above mentioned groups (*ES+JE*, *STO+JE*, *ES+FE*, *STO+FE*) showed any morphological evidence of developing tooth primordia.

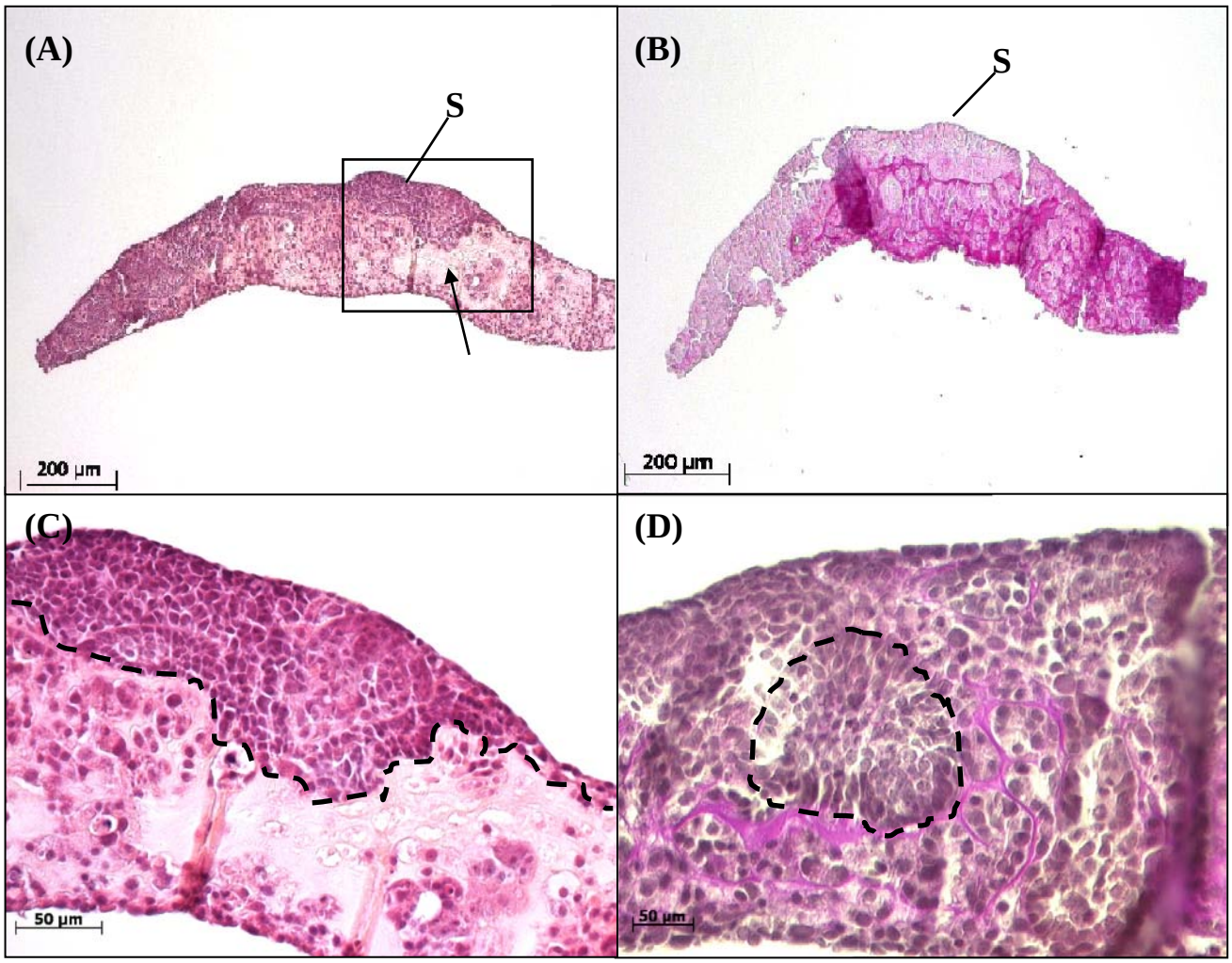

Figure 11: (A) An explant of *ES+FE*, cultured for three days *in vitro*. A cluster of ES cells can be identified by pale eosinophilic extracellular matrix (arrow) surrounding cells with large rounded nuclei (x100). (B) An adjacent section to (A) showing the epithelium on the superficial surface and the PAS-positive ES cell clusters in the deep layers (x100). (C) An enlargement of the area in (A) showing some epithelium (interrupted lines) in the superficial layers of the explant, overlying an ES cell cluster (x400). (D) Cuboidal to columnar epithelium (interrupted lines) can be seen in the deeper layers of the tissue. PAS-positive strands, possibly basement membranes were also observed (x400). S- Superficial surface. (H&E (A and C), PAS (B and D), Bright field microscopy)

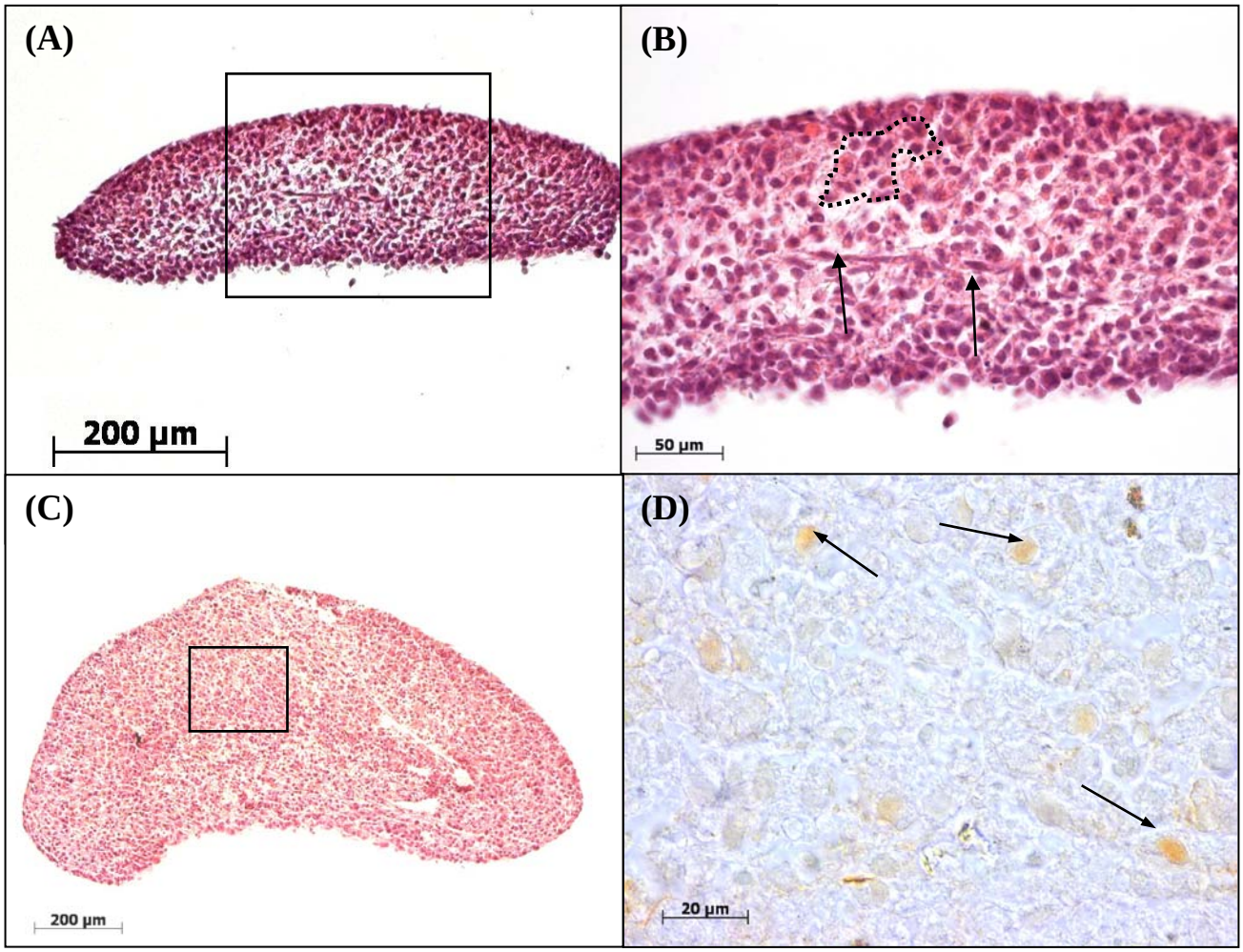

Figure 12: (A,C) Explants of *STO+FE*, cultured for three days *in vitro*. No epithelium is defined (x100). (B) An enlarged area of (A) showing the variation in cell shape and size. The cells in the deeper layers of the explant are more loosely arranged. Some of these cells appear elongated in shape (arrows), while others are more rounded (interrupted lines) (x400). (D) An enlarged area of (C) showing the presence of faint Oct-4 positive nuclei (arrows) (x1000) (H&E (A,B and C), Oct-4 ICC (D), Bright field microscopy)

3.5.3 Histological comparison of explants of *ES+JM*, *STO+JM, ES+FM* and *STO+FM* cultured for three days *in vitro*.

In comparing *ES+JM* (N=13) and *STO+JM* (N=9) explants, the majority of the *ES+JM* explants (N=10) contained ES-like cell clusters visible in the superficial or deeper layers of the tissue (Fig. 13A). The STO cells in four of the *STO+JM* explants (Fig. 14A,B) were present as large cells with intensely eosinophilic and PAS-positive cytoplasm. In four *STO+JM* explants the STO cells could not be distinguished from the ectomesenchymal cells of the lower jaw (Fig. 14C). The ES-like cells were encapsulated by a pale eosinophilic extracellular matrix that also stained positive with the PAS staining technique. In explants of both *ES+JM* (N=6) and *STO+JM* groups (N=4), cuboidal and columnar epithelial cells on seemingly intact basement membranes were visible in the deeper tissue layers (Fig. 13B). Only two *ES+JM* explant contained cuboidal and columnar cells in the superficial layer. Meckel's cartilage was present in seven of the *ES+JM* (Fig. 13B,C,D) and five of the *STO+JM* (Fig. 14) explants. The explants were generally vascularized, although it was often difficult to observe the small capillaries.

Explants of *ES+FM* (N=9) and *STO+FM* (N=9) served as negative control groups for the *ES+JM* and *STO+JM* explants respectively. Compared to the *ES+FE* and *STO+FE* explants, all the *ES+FM* and *STO+FM* explants contained cartilage (Fig.15). The ES cells in the *ES+FM* explants were generally easily distinguishable from the mesenchymal cells by their surrounding pale eosinophilic or PAS-positive extracellular matrix (Fig. 15A,B). STO cells in the *STO+FM* explants (Fig. 15C,D) were present as large cells with intensely eosinophilic and PAS-positive cytoplasm. No epithelium was observed in the superficial layer of any of the *ES+FM* or *STO+FM* explants. In addition, some explants of both the *ES+FM* (N=3) and *STO+FM* (N=4) groups were vascularized.

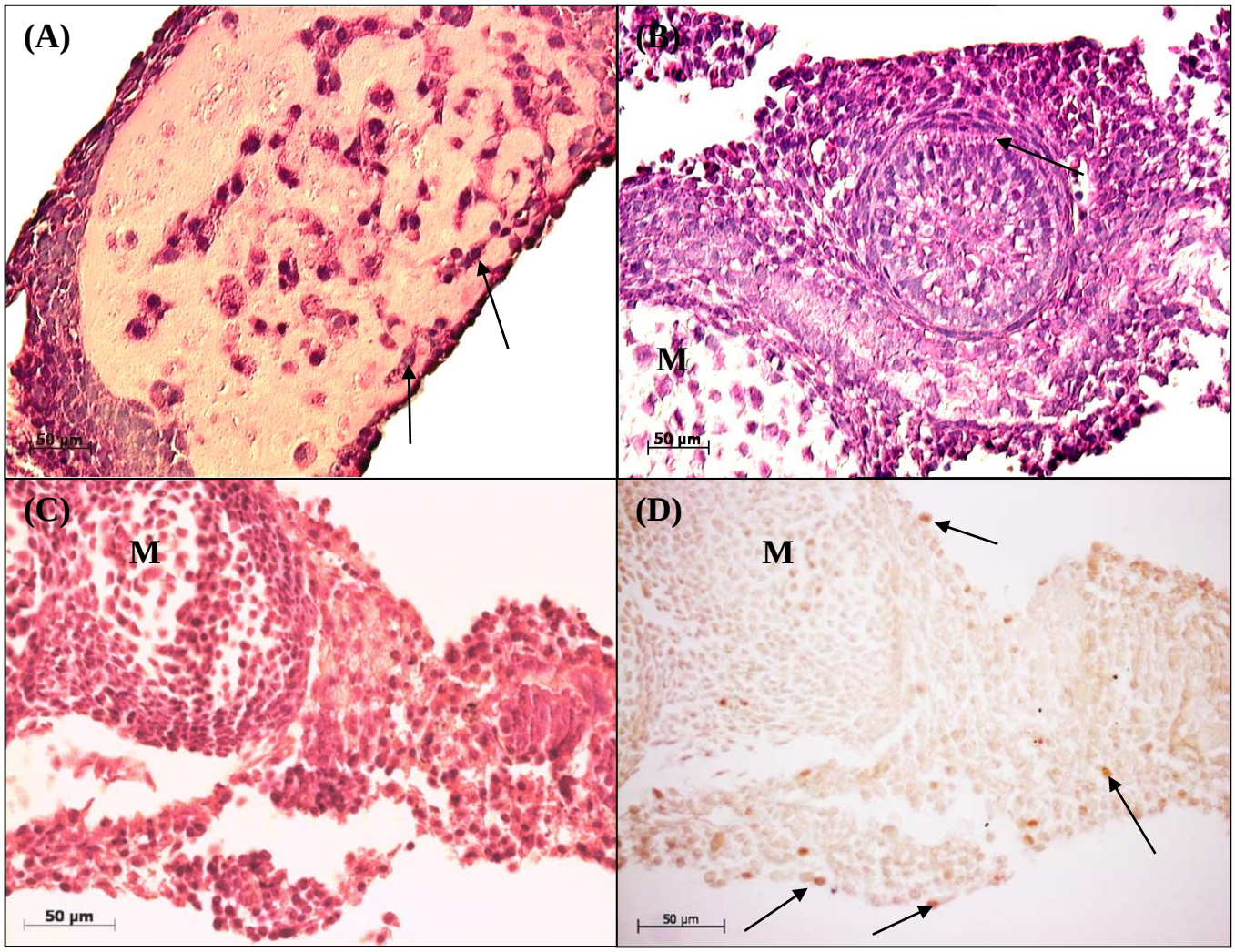

Figure 13: Sections of explants of *ES+JM*, cultured for three days *in vitro*. (A) A large cluster of ES cells in the tissue can be identified by a pale eosinophilic extracellular matrix surrounding large rounded cells. A few of the ES cells (arrows) have flattened out. The densely arranged cells surrounding the ES cell cluster are those of the oral ectomesenchyme (x400). (B) Cuboidal and columnar epithelial cells on seemingly intact basement membranes (arrow) can be seen in the deeper tissue layers (x400). (C) A section of an *ES+JM* explant with an adjacent section (D) showing the presence of Oct-4 positive nuclei (arrows) (x400). M- Meckel's cartilage. (H&E (A,B and C), Oct-4 ICC (D), Bright field microscopy)

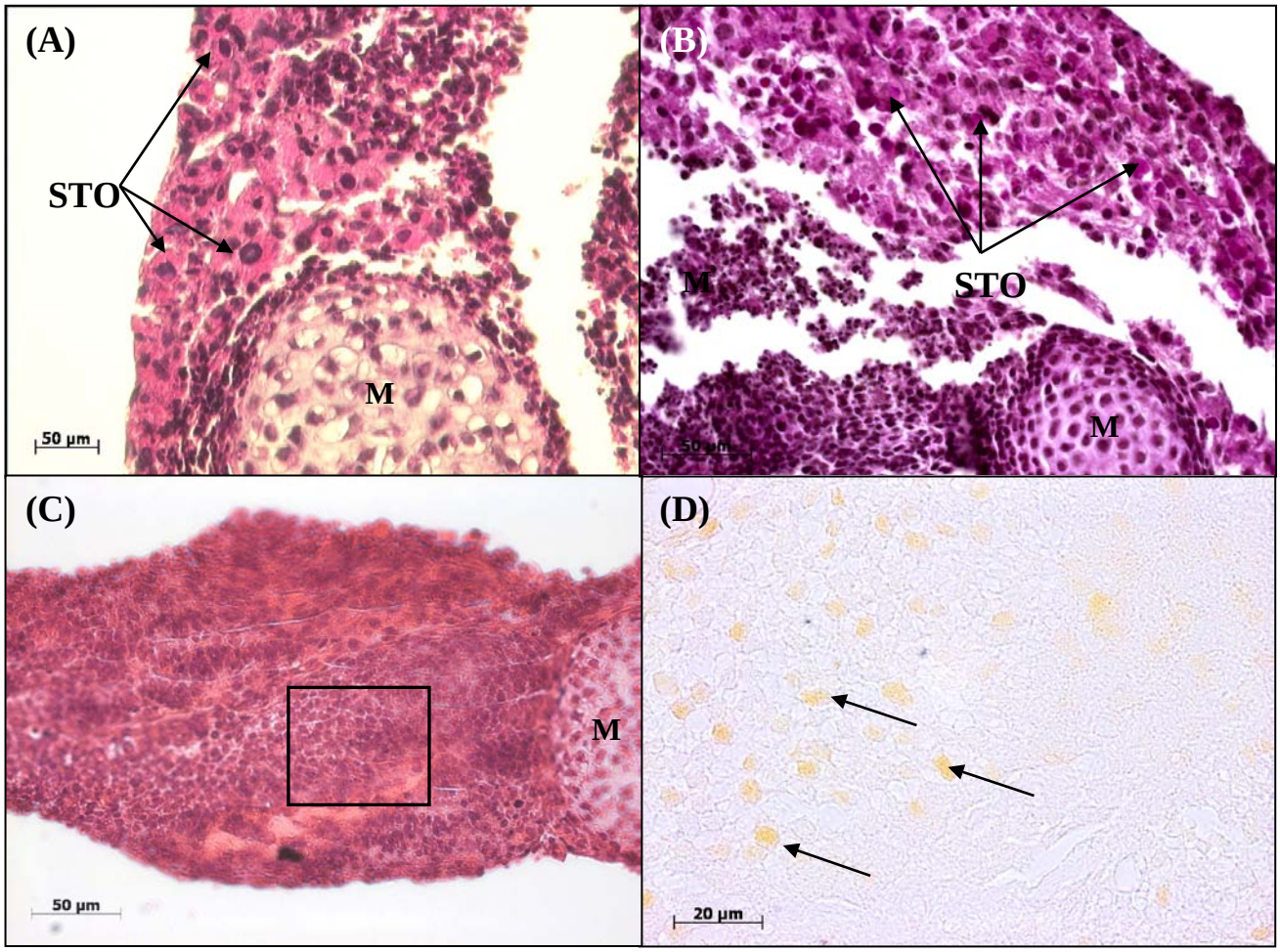

Figure 14: Sections of explants of *STO+JM*, cultured for three days *in vitro*. (A) An explant containing large cells on the superficial surface, presumed to be STO cells (x400). (B) An adjacent section which shows the large cells in (A) stain PAS-positive (x400). (C) A *STO+JM* explant in which the STO cells can not be distinguished from the mesenchyme as all the cells are similar in size (x400). (D) An adjacent section to (C) showing some Oct-4 positive nuclei present in the explant (arrows) (x400). STO- STO cells of a pellet; M- Meckel's cartilage. (H&E (A and C), PAS (B), Oct-4 ICC (D), Bright field microscopy)

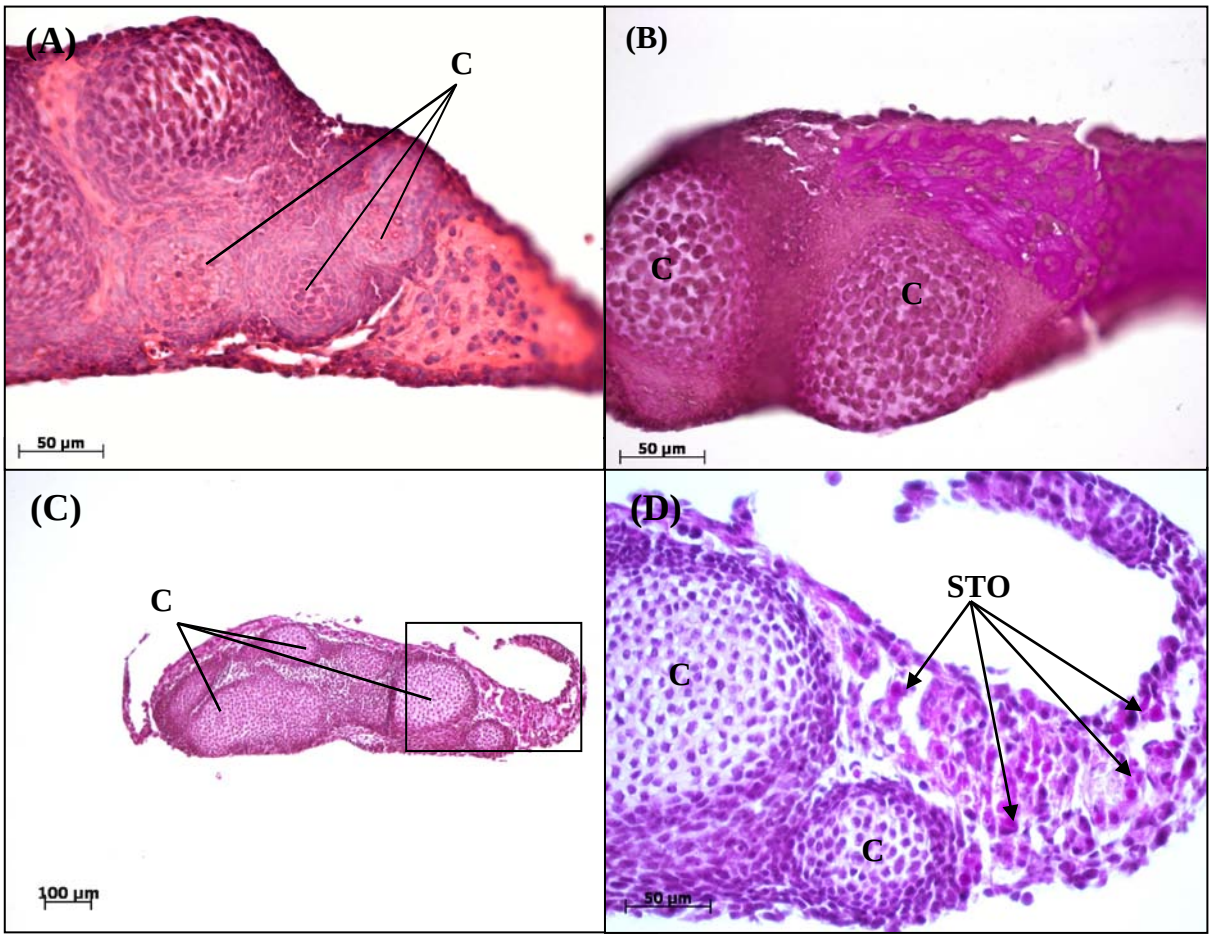

Figure 15: Sections of explants of *ES+FM* (A and B) and *STO+FM* (C and D), cultured for three days *in vitro*. (A) An explant of *ES+FM*, cultured for three days *in vitro.* A large cluster of ES cells in the tissue can be identified by a pale eosinophilic extracellular matrix surrounding rounded cells. Transverse sections of the cartilage of four of the developing digits can also be seen adjacent to the ES cell cluster (x400). (B) An adjacent section showing the same ES cell cluster that has stained PAS-positive (x400). (C) An explant of *STO+FM*, cultured for three days *in vitro* (x100). (D) An enlarged area of (C) showing some STO cells that stain PAS-positive (x400). M- Meckel's cartilage; C- Forelimb cartilage; STO- STO cells of a pellet. (H&E (A and C), PAS (B and D), Bright field microscopy)

3.5.4 Histological comparison of *ES+JE*, *STO+JE, ES+FE*, *STO+FE,* 10,5-day lower jaw and forelimb grafts, cultured for three days *in vitro*, followed by 7 days on the chorio-allantoic membrane of fertile chicken eggs.

The culturing of grafts on the chorio-allantoic membrane of fertile chicken eggs did not yield a very large sample size. Nine grafts were dead upon retrieval. None of the cultured lower jaws (N=5) and forelimbs (N=5), *ES+FE* grafts or *STO+JE* grafts survived. Five of the thirteen *ES+JE* grafts that were cultured survived (Table 1). Of the five *STO+FE* grafts that were cultured, four grafts were retrieved, of which two had survived culturing (Table 1).

Histological analysis of the *ES+JE* grafts showed that four of the grafts contained well-defined stratified squamous keratinized epithelium, which were wrapped around centrally placed ES cell clusters (Fig. 16A,C). These ES cell clusters contained numerous mitotic cells in a pale eosinophilic, as well as PAS-positive extracellular matrix. The cells toward the periphery of the clusters were flattened compared to those in the centre of the clusters (Fig. 16D). A PAS-positive basement membrane was visible underlying the superficial keratinized epithelium in sections of some grafts (Fig. 16B). The *ES+JE* grafts also had few blood vessels present, although many of these seemed to be host blood vessels that had infiltrated the grafts. The *ES+JE* grafts did not have any hair follicles or glands present. In addition, mitotic figures were observed in some of the ES cells.

Both of the *STO+FE* grafts that survived the culturing period showed the presence of many fibroblast-like cells (Fig. 17A,B). A few blood vessels were present. These were of host origin, as was identified by the elliptical shape of the chick erythrocytes and their nuclei (Scott, 1966; Barrett and Dawson, 1974).

3.5.5 Histological comparison of *ES+JM*, *STO+JM, ES+FM*, *STO+FM,* 12,5-day lower jaw and forelimb grafts, cultured for three days *in vitro*, followed by 7 days on the chorio-allantoic membrane of fertile chicken eggs.

Three lower jaws survived the culturing period (Table 2). Haematoxylin and eosin sections of these grafts showed the presence of Meckel's cartilage, surrounded by fibroblast-like cells and some keratinized epithelium. Host blood vessels were observed in two of the grafts. Possible signs of apoptosis were also present in two of the grafts. No tooth formation was observed. The two surviving forelimb grafts contained cartilage, mesenchyme and many host blood vessels. One of the grafts also contained some keratinized epithelium in the superficial and deep layers of the tissue.

While nine of the ten *ES+JM* grafts contained Meckel's cartilage, the *STO+JM* graft (N=1) contained no cartilage. The *STO+JM* graft consisted of a mass of small rounded cells, surrounded by a few loosely arranged fibroblasts. There was epithelium present on the superficial surface of nine of the *ES+JM* grafts (Fig. 18A), as well as the *STO+JM* graft (Fig. 18C). The *ES+JM* grafts also contained some stratified squamous keratinized and non-keratinized epithelium in the deeper layers, as well as small clusters of what appeared to be ES cells surrounded by a PAS-positive extracellular matrix (Fig. 18B). The cells at the periphery of these clusters were similar to those of the *ES+JE* grafts and appeared flattened and smaller than the cells in the centre of the clusters. No basement membrane was identified in the *STO+JM* graft. The *ES+JM* grafts in addition were very vascular and two of these grafts had hair follicles in isolated areas, whereas no blood vessels or hair follicles were observed in the *STO+JM* graft.

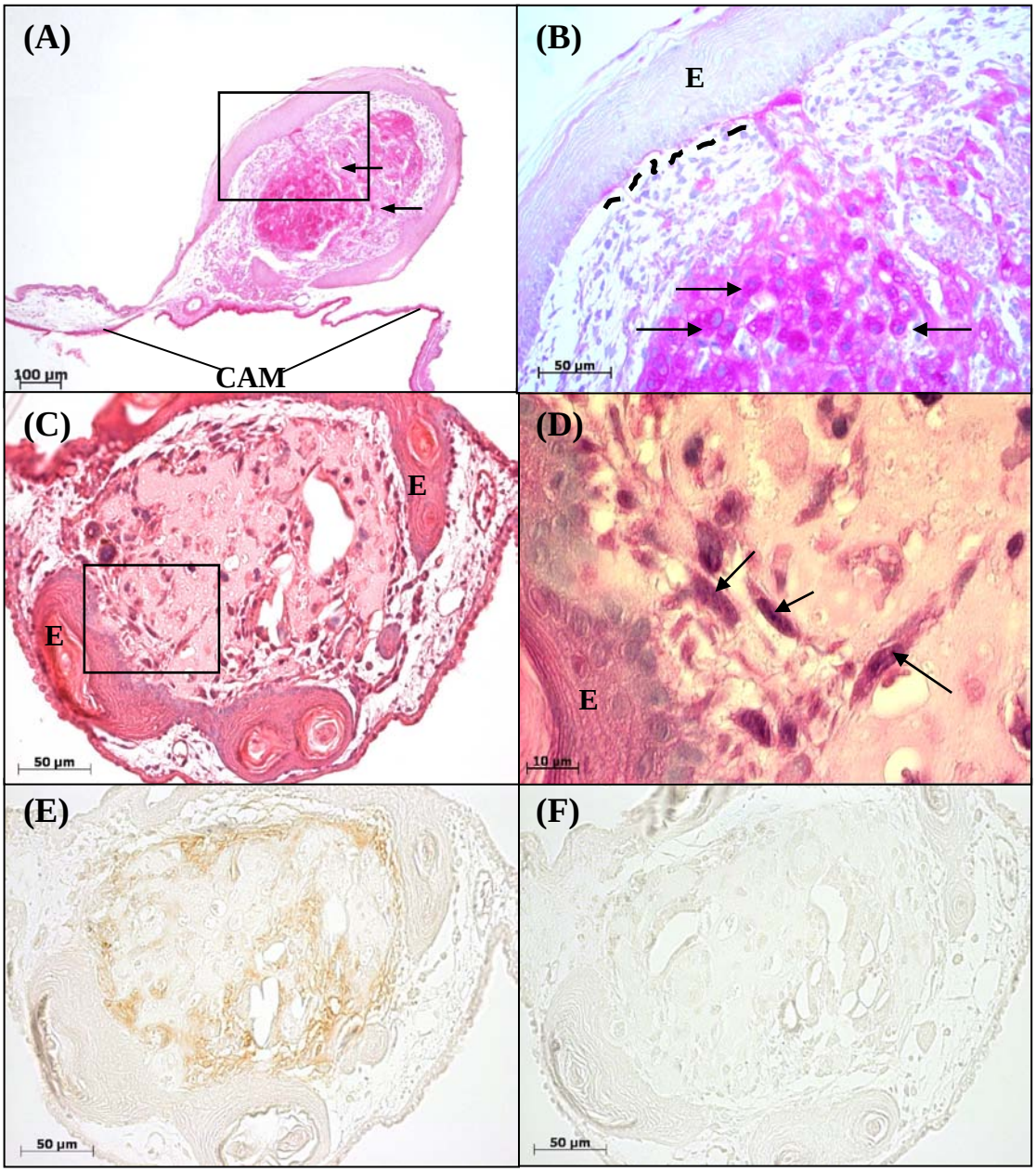

Figure 16: Grafts of *ES+JE*, cultured for three days *in vitro,* followed by 7 days on the CAM of fertile chicken eggs. (A) Stratified squamous keratinized epithelium can be seen surrounding the centrally placed PAS-positive ES cell clusters. Large sections of the graft are infiltrated with blood vessels of the host CAM (arrows) (x100). (B) An enlarged view of the marked area in (A) showing the ES cells with large rounded basophilic nuclei and PAS-positive cytoplasm (arrows). A disrupted PAS-positive basement membrane is indicated be the interrupted line (x400). (C) Stratified squamous keratinized epithelium also surrounds this centrally placed eosinophilic extracellular matrix of the ES cell cluster (x400). (D) An enlarged view of the marked area in (C) showing the elongated ES cells with flattened nuclei (arrows) at the periphery of the ES cell cluster (x600). (E) An adjacent section to (C) showing the Oct-4 positive labeled periphery of the ES cell cluster (x400). (F) A negative control section adjacent to (E) showing no label (x400) The dark areas in this image is as a result of raised keratinized tissue, no trapping was present. E- Stratified squamous keratinized epithelium; CAM- Chorio-allantoic membrane of the host egg. (H&E (C and D), PAS (A and B), Oct-4 ICC (E and F), Bright field microscopy)

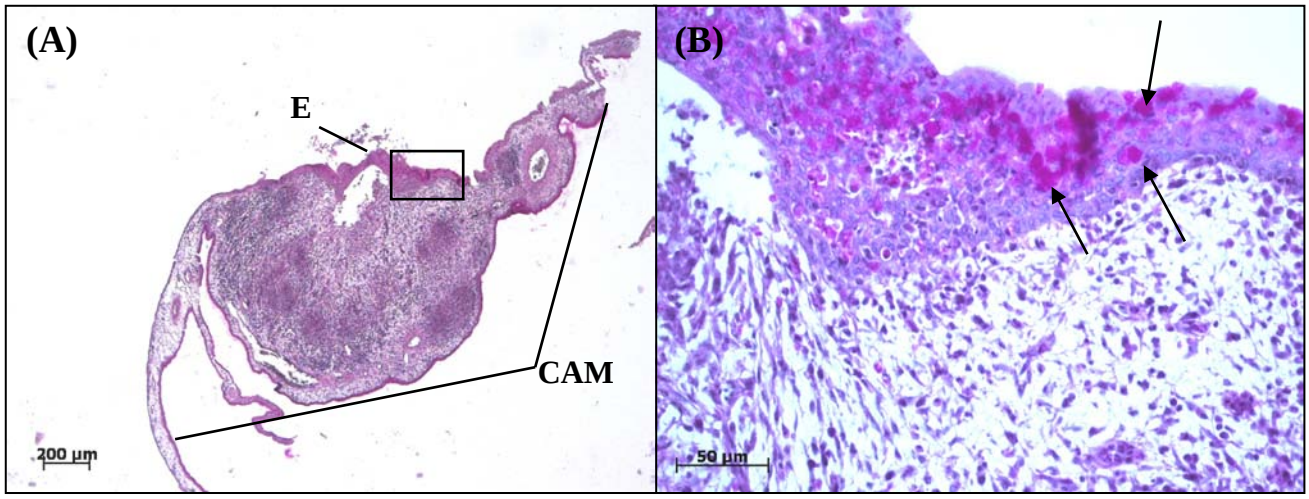

Figure 17: Grafts of *STO+FE*, cultured for three days *in vitro,* followed by 7 days on the CAM of fertile chicken eggs. (A) A large mass of cells can be seen encapsulated by the CAM of the host egg (x50). (B) An enlarged view of the marked area in (A) showing some PAS-positive epithelial cells (arrows) of a stratified, non-keratinized epithelium superficial to the STO cell mass (x400). E- Stratified squamous non-keratinized epithelium; CAM- Chorio-allantoic membrane of the host egg. (PAS, Bright field microscopy)

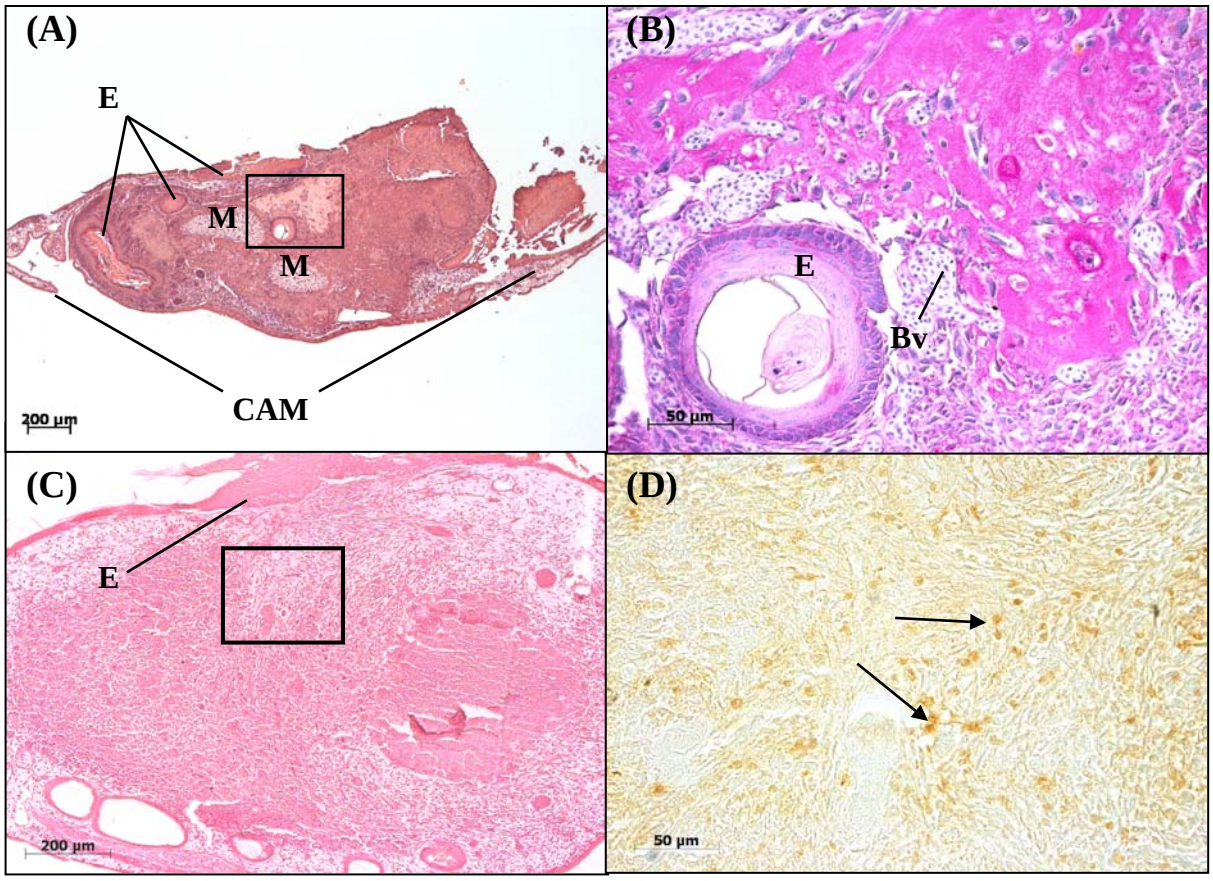

Figure 18: Grafts of *ES+JM* and *STO+JM*, cultured for three days *in vitro,* followed by 7 days on the CAM of fertile chicken eggs. (A) An *ES+JM* graft containing Meckel's cartilage, some stratified squamous keratinized epithelium and hair follicles (x50). (B) An adjacent section to (A), showing an enlarged view similar to the marked area in (A). A large PAS-positive extracellular matrix is visible adjacent to some stratified squamous keratinized epithelium (x400). (C) A *STO+JM* graft showing a large mass of cells surrounded by the CAM of the host egg. Some Stratified epithelium is present on the superficial surface of the graft (x50). (D) An enlarged view of the marked area in (C) showing the presence of cells with Oct-4 positive nuclei (arrows) (x400). E- Stratified squamous epithelium; CAM- Chorio-allantoic membrane of the host egg; M- Meckel's cartilage; Bv- blood vessel. (H&E (A and C), PAS (B), Oct-4 ICC (D), Bright field microscopy)

Grafts of *ES+FM* and *STO+FM* served as negative control groups for the *ES+JM* and *STO+JM* grafts respectively. All the *ES+FM* (N=4) and *STO+FM* (N=2) grafts contained cartilage. Some squamous keratinized epithelium was present in two *ES+FM* grafts and one *STO+FM* graft. ES cell clusters were only identified in two of the *ES+FM* grafts by the presence of a pale eosinophilic extracellular matrix that surrounded these cells. The *STO+FM* grafts were less vascularized than the *ES+FM* grafts.

3.5.6 Histological comparison of *ES+JE, STO+JE* and 10,5-day lower jaw grafts, cultured for three days *in vitro*, followed by 21 days under the renal capsules of adult male mice.

Six of the seven cultured 10,5-day lower jaw halves, which were cultured as RS grafts, survived and continued their developmental fates to some extent (Table 1). All six of these grafts contained fragments of osteogenic tissue (bone) and some loose connective tissue (Fig. 19C). One of these grafts contained a well-developed molar (Fig. 19A). Five of the grafts contained blood vessels. Two grafts contained skeletal muscle fibres, which were presumed to be of ectomesenchymal origin. Two grafts contained numerous adipocytes, as well as keratinized squamous epithelium and glands adjacent to hair follicles (Fig. 19A,B).

Six of the seven *ES+JE* RS grafts survived and developed (Table 1). All six of these grafts however, seemed to display features of cystic tissue. All the grafts were very large in size upon retrieval, ranging from 5mm^3 to 15mm^3. All six grafts contained ciliated cuboidal and columnar epithelium with PAS-positive, diastase resistant mucous cells in the deeper layers of the tissue (Fig. 20A,C). The mucinous epithelium lined lumina

contained PAS-positive secretions and cellular debris. Little pale eosinophilic or PAS-positive extracellular matrix was present throughout the grafts. Keratinized squamous epithelium was observed in the deep layers of five of the grafts. No tooth formation was observed. Numerous neutrophils were observed in all of the *ES+JE* grafts, as well as giant, multinucleated cells with PAS-positive, diastase resistant granules in their cytoplasm (Fig. 20F). These cells appeared to be megakaryocytes. Three of the grafts contained a gelatinous yellow-brown substance.

Three of the grafts contained what appeared to resemble either cardiac or skeletal muscle with striations and central to eccentrically placed nuclei, which made up the bulk of the tissue (Fig. 20A,C,E). Staining sections of these grafts with Verhoeff's iron haematoxylin revealed no intercalated discs. These striated fibres were thus that of developing skeletal muscle (Fürst et al., 1989). Two of these grafts also contained fragments of cartilage and bone (Fig. 20A). Three of the *ES+JE* graft contained many thick bands of what seemed to be smooth muscle cells, which made up the bulk of the tissue (Fig. 20B,D).

Three *STO+JE* grafts were transplanted under the renal capsules of adult mice. None of these grafts survived.

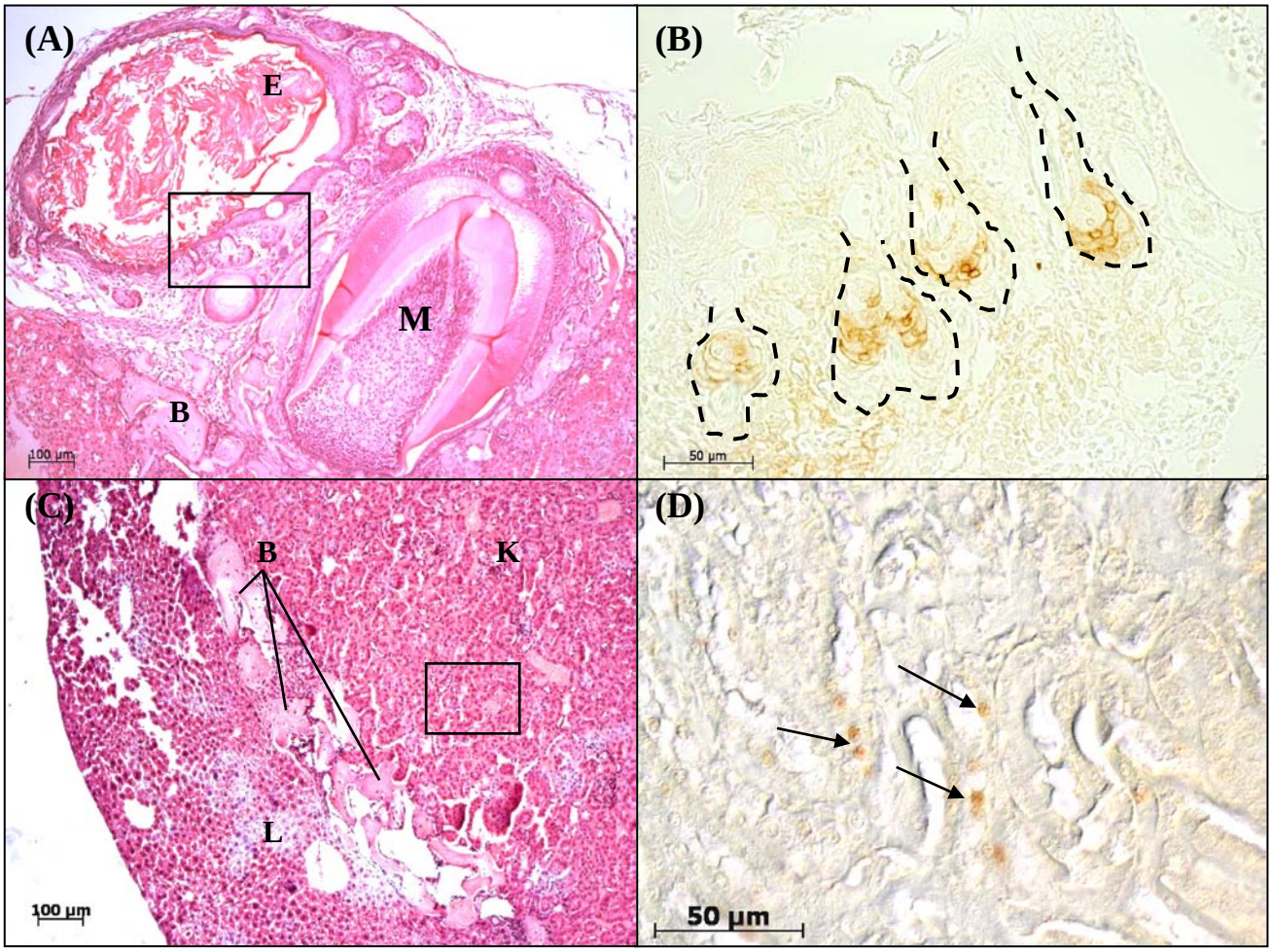

Figure 19: Grafts of 10,5 day lower jaws, cultured for three days *in vitro,* followed by 21 days under the renal capsules of mice. (A) A graft containing a well developed molar, bone, stratified squamous keratinized epithelium, connective tissue and sebaceous glands (x100). (B) An enlarged view of the marked area in (A) showing positive Oct-4 labeled cells of the sebaceous glands (outlined) (x400) (C) A graft that contains only fragments of bone (x100). (D) An enlarged view of the marked area in (C) showing Oct-4 positive nuclei in the epithelial cells of the collecting tubules of the host kidney (x1000). E- stratified squamous keratinized epithelium; B- bone; M- molar; K- parenchyma of kidney; L- liver. (H&E (A and C), Oct-4 ICC (B and D), Bright field microscopy)

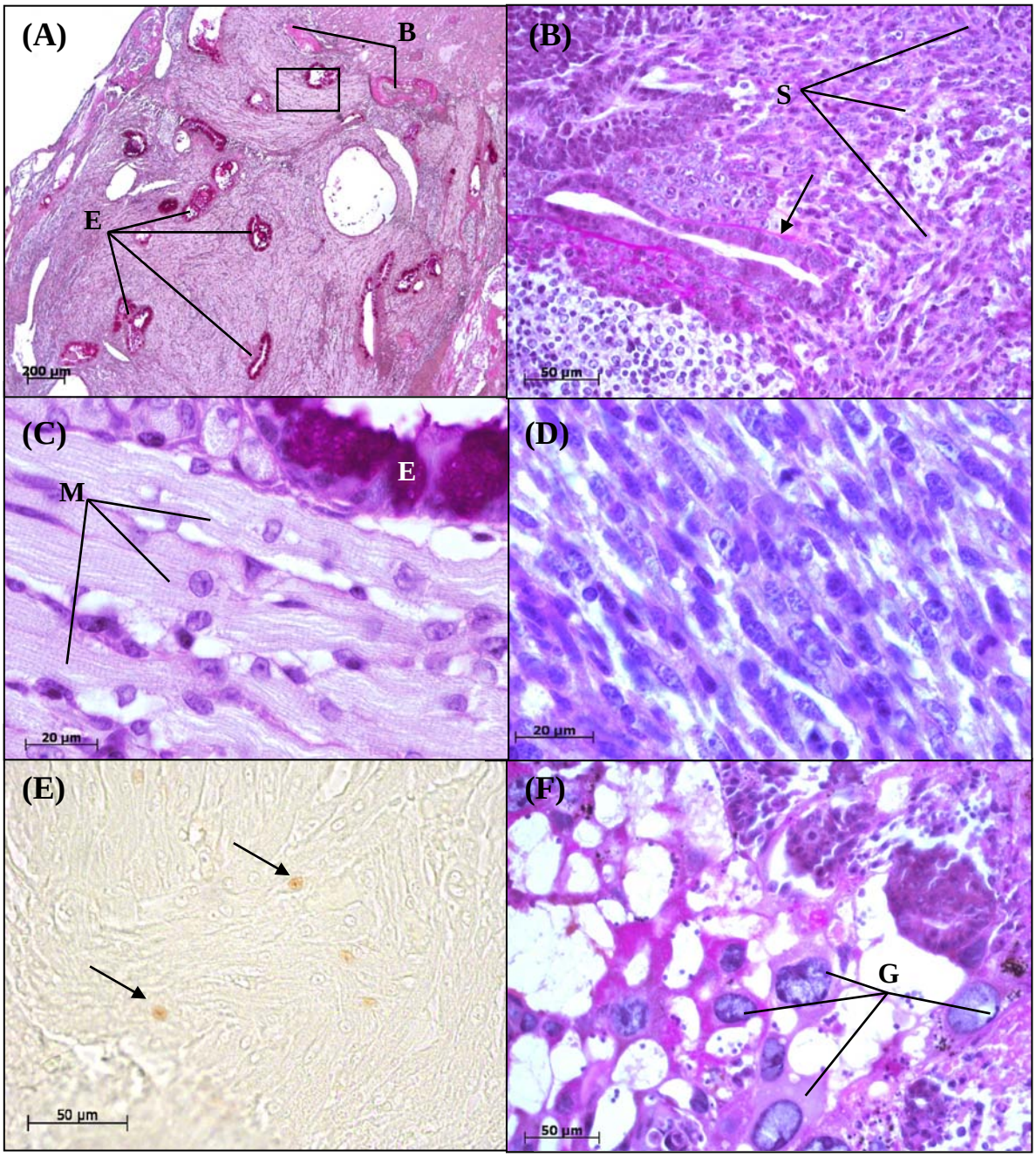

Figure 20: Grafts of *ES+JE*, cultured for three days *in vitro,* followed by 21 days under the renal capsules of mice. (A) A graft consisting mainly of skeletal muscle fibres. Some PAS-positive mucinous epithelium is present as well as fragments of bone (x50). (B) A graft consisting mainly of smooth muscle cells. The graft also contains some epithelium on a PAS-positive basement membrane (arrow), surrounded by smooth muscle cells (x400). (C) An enlarged view of the marked area in (A) showing the PAS positive mucous epithelium surrounded by developing skeletal muscle fibers (x1000). (D) An enlarged view of the smooth muscle cells found in (B) (x1000). (E) Oct-4 positive nuclei of some of the muscle fibers found in (A) (x400). (F) Giant cells with large rounded basophilic nuclei and PAS-positive, diastase-resistant granules in their cytoplasm (x400). E- mucous cuboidal to columnar epithelium; B- bone; M- skeletal muscle fibres; S- smooth muscle cells; G- giant cells. (PAS (A,B,C,D and F), Oct-4 ICC (E), Bright field microscopy)

3.5.7 Histological comparison of *ES+JM, STO+JM* and 12,5-day lower jaw grafts, cultured for three days *in vitro*, followed by 21 days under the renal capsules of adult male mice.

All five of the cultured 12,5-day lower jaw halves that were transplanted under the renal capsules of adult mice, survived and developed (Table 2). All of these grafts contained bone, Meckel's cartilage, adipose tissue, some keratinized squamous epithelium with hair follicles and glands (Fig. 21A). A few skeletal muscle fibres were observed in two of the grafts. Two of the grafts each contained two well-developed molars, which had formed predentine and enamel (Fig. 21B). One of these grafts also contained a well-developed incisor (Fig. 21A). One graft contained one well developed molar. One graft did not contain any recognizable teeth, but instead showed some features of cystic tissue. The graft contained simple ciliated cuboidal and columnar epithelium with mucous cells. There were also numerous lymphocytes present in the mesenchymal tissue.

Of the twelve *ES+JM* grafts that were transplanted under the renal capsules of adult male mice, eleven were retrieved. One of these grafts however, only consisted of a few fragments of osteogenic tissue and some loose eosinophilic fibres. Ten of the grafts showed some further development (Table 2). These grafts all contained bone, adipose tissue and keratinized squamous epithelium. Three grafts also contained some cartilage. Four of the grafts contained hair follicles and sebaceous glands. Neutrophils and lymphocytes were also observed in most of the grafts. Two of the grafts each contained two well-developed molars that had formed dentine and enamel (Fig. 22A,C). One graft contained one incisor and one molar (Fig. 22B).

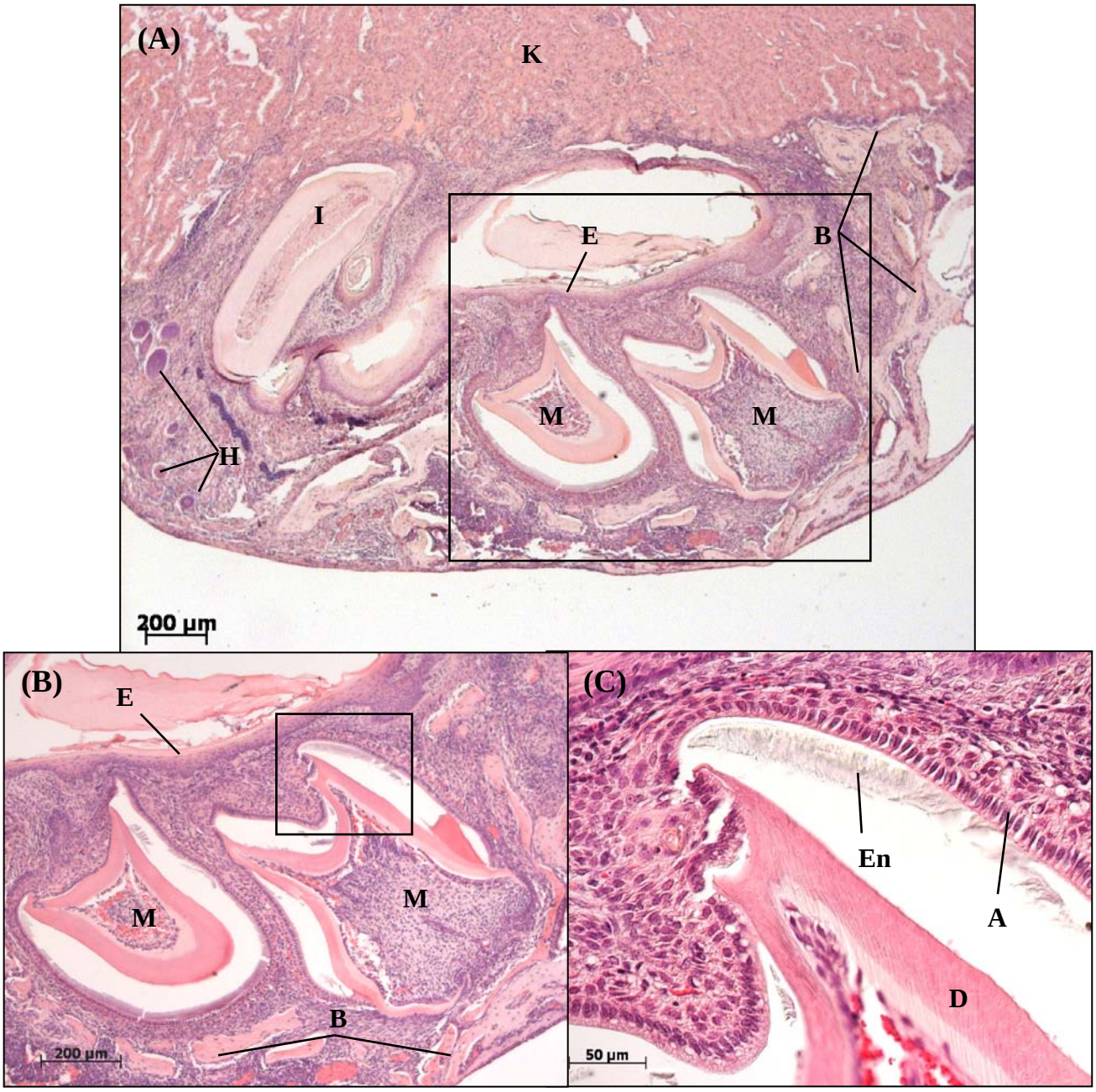

Figure 21: Grafts of 12,5 day lower jaw halves, cultured for three days *in vitro,* followed by 21 days under the renal capsules of mice. (A) A graft containing an incisor, two molars, bone, stratified squamous keratinized epithelium, connective tissue and hair follicles (x50). (B) An enlarged view of the marked area in (A) showing the two molars (x100). (C) An enlarged view of the marked area in (B) showing the ameloblasts of the molar, the predentin and forming enamel (x400). E- stratified squamous keratinized epithelium; B- bone; I- incisor; M- molar; H- hair follicle; K- parenchyma of kidney; A- ameloblasts; D- dentin; En- enamel. (H&E, Bright field microscopy)

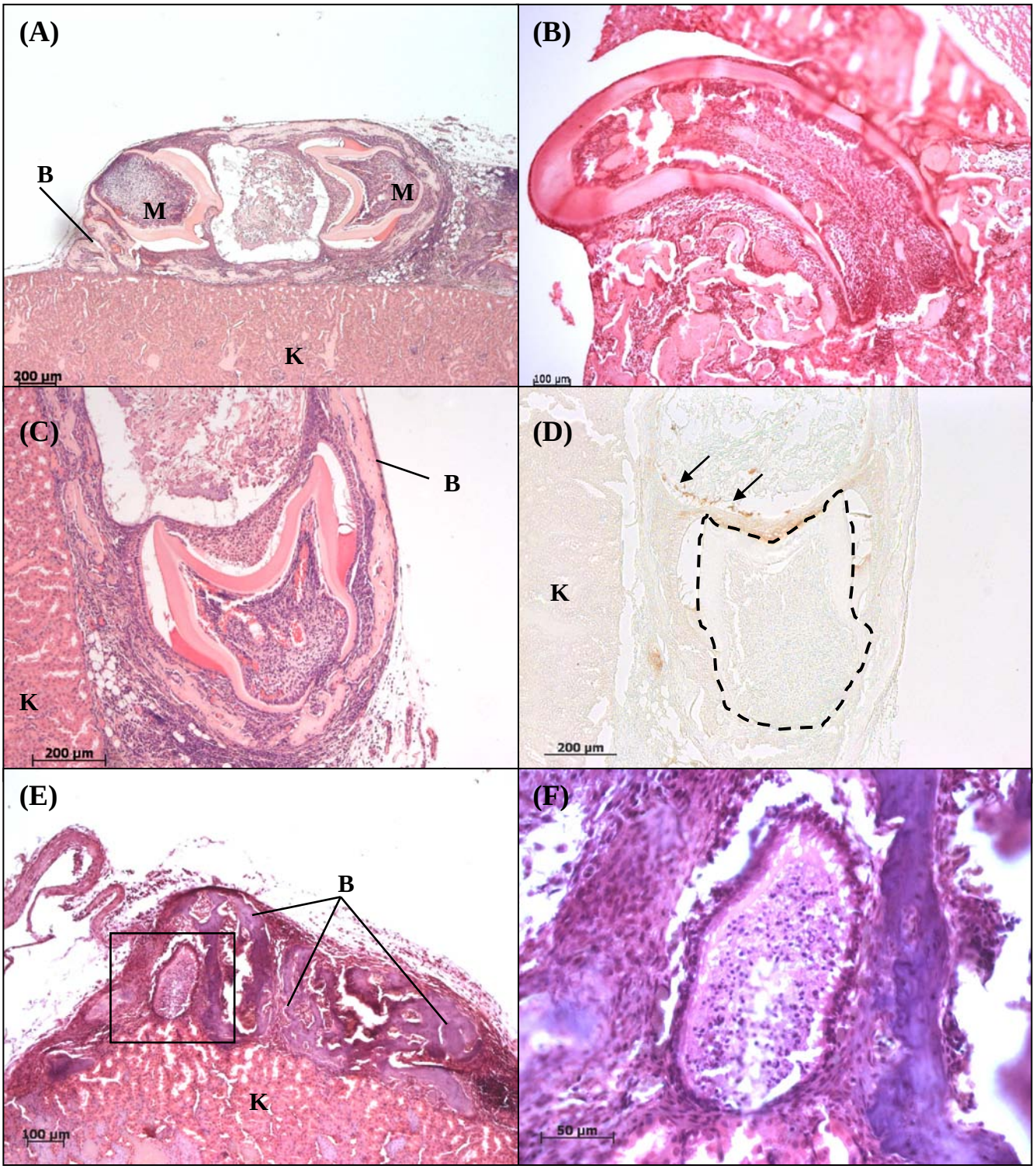

Figure 22: Grafts of *ES+JM*, cultured for three days *in vitro,* followed by 21 days under the renal capsules of mice. (A) A graft containing two molars, bone and connective tissue (x50). (B) A graft containing an incisor (x100). (C) An enlarged view of one of the molars in (A) (x100). (D) An adjacent section to (C) showing Oct-4 positive labeled nuclei (arrows) in the lumen and fibers in the enamel organ superficial to the developing cusps (x100). (E) A graft containing one tooth, bone and connective tissue (x100). (F) An enlarged view of the marked area in (E) showing what appears to be degeneration occurring in the layers of the tooth (x400). B- bone; M- molar; K- parenchyma of kidney. (H&E (A,B,C,E and F), Oct-4 ICC (D), Bright field microscopy)

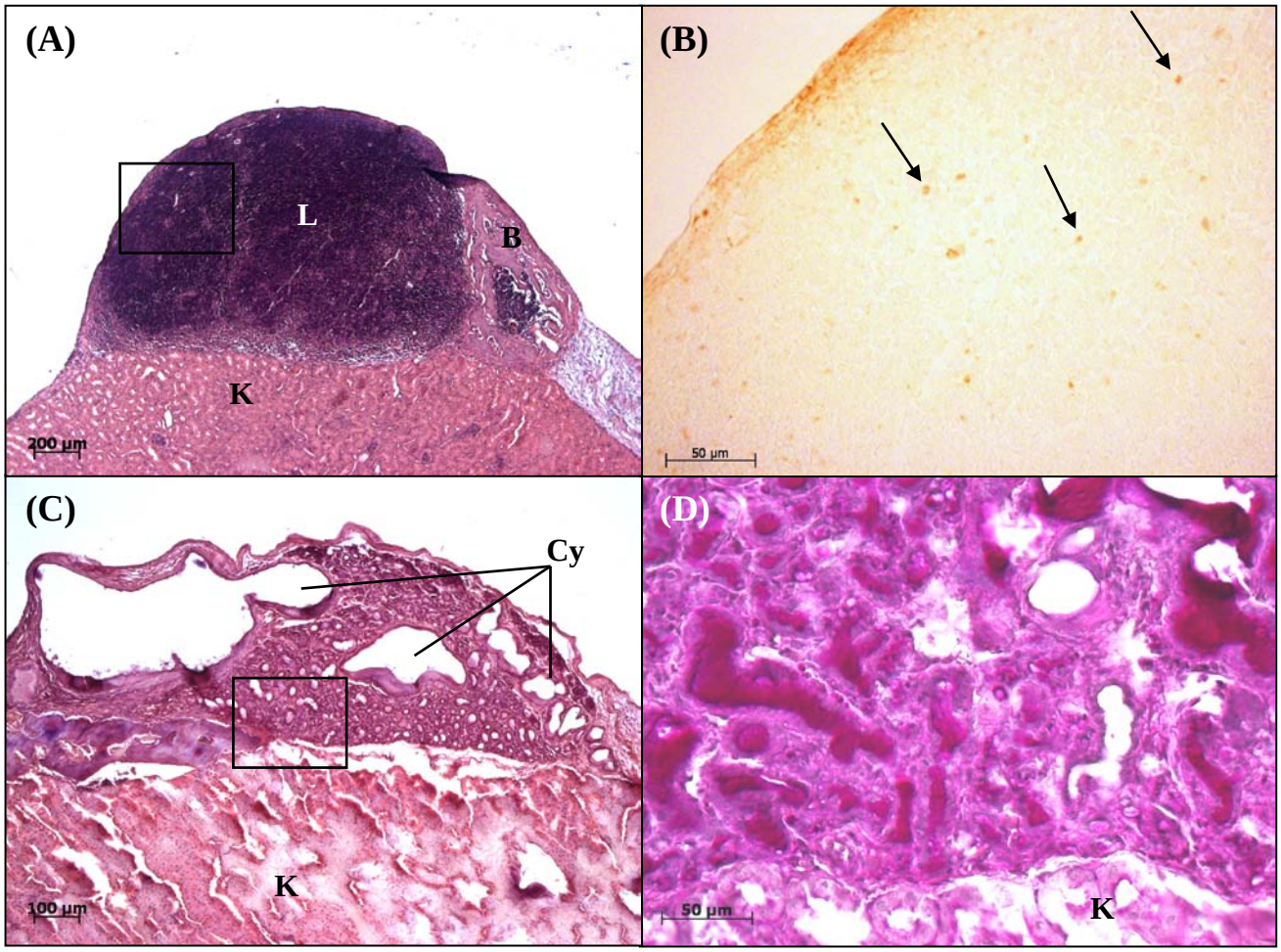

Figure 23: Grafts of *STO+JM*, cultured for three days *in vitro,* followed by 21 days under the renal capsules of mice. (A) A graft containing bone and bone marrow, some connective tissue, and a large aggregation of lymphocytes (x50). (B) An adjacent section to (A), showing an enlarged view similar to the marked area in (A). Oct-4 positive nuclei are present throughout the aggregation of lymphocytes (arrows) (x400). (C) A graft containing bone, connective tissue, large cyst-like structures and small duct-like structures (x100). (D) An adjacent section to (C), showing an enlarged view similar to the marked area in (C). Some PAS-positive staining is visible in the lumina of the small duct-like structures (x400). B- bone; L- lymphocytes; K- parenchyma of kidney; Cy- cyst-like structures. (H&E (A and C), Oct-4 ICC (B), PAS (D), Bright field microscopy)

One graft contained a smaller developing tooth, which showed some predentine formation, but also signs of cellular degeneration (Fig. 22E,F). One graft contained what appeared to be cyst-like fluid filled structures lined by cuboidal cells, as well as numerous small ducts lined by cuboidal cells in the deeper layers of the tissue. Four grafts appeared to be cystic and contained numerous neutrophils and grouped giant, multinucleated cells with PAS-positive, diastase resistant granules in their cytoplasm.

Five *STO+JM* grafts were transplanted under the renal capsules of adult male mice. Four of the grafts were retrieved (Table 2). All of the grafts contained bone, lymphocytes and adipose tissue. Two grafts contained some keratinized squamous epithelium with sebaceous glands and hair follicles that were possibly included with the mesenchyme. One graft showed a few skeletal muscle fibres as well as a large aggregate of what appeared to be lymphocytes (Fig. 23A,B). One graft contained large cyst-like structures and numerous small duct-like structures lined by cuboidal cells in the deeper layers of the tissue (Fig. 23C,D). No morphological evidence of tooth formation was observed in any of the *STO+JM* grafts.

3.6 Immunological analysis

3.6.1 Oct-4 labelling

Immunolabelling for Oct-4 was performed on sections of all explants and grafts containing ES cells. To determine the specificity of the Oct-4 label to undifferentiated ES cells, sections of explants and grafts containing STO cells were also labelled for Oct-4.

Although Oct-4 is a nuclear label, some Oct-4 labelling was also seen in extracellular fibres present throughout the explants and grafts. Nuclear and fibrous Oct-4 labelling was observed in the three-day cultured *ES+JE, ES+FE, ES+JM* (Fig. 13D) and *ES+FM* explants. Faint Oct-4 positive nuclei and fibres were also observed in the *STO+FE* (Fig. 12D) and *STO+FM* explants, while only a nuclear label was seen in the *STO+JM* explants (Fig. 14D). The *ES+JE* (Fig. 16E,F)*, ES+JM, ES+FM* and *STO+JM* (Fig. 18D) CAM grafts, and the *ES+JE* (Fig. 20E) and *ES+JM* (Fig. 22D) RS grafts also contained Oct-4 positive nuclei and fibres (For numbers, see Table 1 and 2). One of the *STO+JM* RS grafts appeared to consist largely of an aggregation of lymphocytes, which also contained some faint Oct-4 positive nuclei (Fig. 23A,B). Oct-4 positive nuclei were observed in the sebaceous glands present in the cultured lower jaw RS grafts of both age groups (Fig. 19B). All the RS grafts showed faintly labelled Oct-4 positive nuclei in the epithelium of the kidney tubules (Fig 19D). No Oct-4 positive nuclei or extracellular fibres were observed in the negative control sections.

3.6.2 Msx1 labelling

The Msx1 immunolabelling could not be optimised and was therefore excluded from the study.

Table 1: Summary of the number of cultured 10,5-day explants and grafts of each group that were retrieved, that survived the culture period and the morphological features observed in sections of the tissue obtained

Type of culturing method & duration	Tissue combinations	No. cultured	No. retrieved	No. that survived culturing	Percentage that survived culturing	Summary of the number of grafts that exhibit certain morphological features															
						ECM of ES cell clusters	Cartilage	Mesenchyme/ Soft tissue	Supperficial epithelium	Deep epithelium	Blood vessels	Apoptosis	Tooth	Bone	Cystic tissue	Muscle cells	PAS$^+$ granules	PAS$^+$ fibres	PAS$^+$ ECM of ES cell clusters	Oct-4$^+$ cell nuclei	Oct-4$^+$ extracellular fibres
3 days *in vitro*	Whole jaw	9	7	4	44			4	4	2	1	1					2	1			
	Whole forelimb	15	8	5	33			4	4	3	2	2					2				
	ES+JE	11	8	7	64	7			7	7		7					3	5	4	2	2
	ES+FE	10	7	7	70	7			7	7	1	5					4	5	5	3	1
	STO+JE	14	12	7	50							6					4				
	STO+FE	12	9	9	75							8					7			3	2
3 days *in vitro* + 7 days CAM	Whole jaw	5	1	0	0																
	Whole forelimb	5	1	0	0																
	ES+JE	13	8	5	38	5		5	5	5	4	1					4	3	3	2	2
	ES+FE	4	1	0	0																
	STO+JE	5	0	0	0																
	STO+FE	5	4	2	40			2	2	2		2									2
3 days *in vitro* + 21 days RSG	Whole jaw	7	6	6	86		3	6	6	5	6		1	6	1	2				1	
	ES+JE	7	6	6	86	4	2	6	6	6	6	3		2	6	6	5	5	5	4	3
	STO+JE	4	4	0	0																

Table 2: Summary of the number of cultured 12,5-day explants and grafts of each group that were retrieved, that survived the culture period and the morphological features observed in sections of the tissue obtained

Type of culturing method & duration	Tissue combinations	No. cultured	No. retrieved	No. that survived culturing	Percentage that survived culturing	Summary of the number of grafts that exhibit certain morphological features															
						ECM of ES cell clusters	Cartilage	Mesenchyme/ Soft tissue	Supperficial epithelium	Deep epithelium	Blood vessels	Apoptosis	Tooth	Bone	Cystic tissue	Muscle cells	PAS$^+$ granules	PAS$^+$ fibres	PAS$^+$ ECM of ES cell clusters	Oct-4$^+$ cell nuclei	Oct-4$^+$ extracellular fibres
3 days *in vitro*	Whole jaw	20	18	15	75		11	15	15	3	15	3					7	5			
	Whole forelimb	11	10	10	91		9	10	5		4	2					3	3			
	ES+JM	21	17	13	62	12	7	13	1	7	8	5					10	5	10	11	2
	ES+FM	12	11	9	75	9	5	9		1	4	1					4	4	2	5	
	STO+JM	18	12	9	50		5	9	4	4	5	6					5	4		2	
	STO+FM	14	11	9	64		9	9		3	4	1					1			3	1
3 days *in vitro* + 7 days CAM	Whole jaw	6	3	3	50		1	3	1	1	3	2									
	Whole forelimb	5	2	2	40		2	2	1	1	2										
	ES+JM	15	13	10	67	9	9	10	8	8	10	6					4	6	2	7	3
	ES+FM	5	4	4	80	2	4	4	2		4	1		1			1	1		2	1
	STO+JM	8	5	1	13			1	1	1	1						1			1	1
	STO+FM	4	3	2	50		2	2	2		2	1					3				
3 days in vitro + 21 days RSG	Whole jaw	5	5	5	100		4	5	5	1	5		3	5	1	1	1	1		1	1
	ES+JM	12	11	10	83		4	10	5		10	2	4	10	4	2	3	2	2	3	5
	STO+JM	5	4	4	80			4	2	1	4			4	1	1				1	

4. DISCUSSION

4.1 The renal subcapsular grafts

This study aimed to determine whether embryonic stem cells were able to differentiate and contribute to tooth formation when combined with mouse embryonic lower jaw ectomesenchyme *in vitro* and *in vivo*. The results obtained showed that four of the ten *ES+JM* RS grafts that survived the culture period developed teeth. In three of the grafts, the teeth had developed to stages comparable to those observed in the control 12,5-day jaw RS grafts. One *ES+JM* contained a small developing tooth that may have been undergoing degeneration. However, it has been shown that when tooth germs are cut into half and cultured, each half will form a complete tooth, but one that is smaller than a whole cultured tooth (Glasstone, 1952). It is also possible that some of the mesenchymal tissue had been removed with the dental epithelium during the initial separation of the tissue layers, which may have resulted in the smaller tooth that was observed.

All the control *STO+JM* grafts survived the culture period and developed structures such as bone, muscle and hair. However, no teeth were formed in these grafts, which may imply that the inclusion of ES cells with the lower jaw ectomesenchyme could possibly have contributed to tooth formation in the *ES+JM* grafts.

None of the *STO+JE* RS grafts developed, whereas six out of seven *ES+JE* grafts survived and developed muscle and mucinous epithelium, with large aggregations of lymphocytes in some of the grafts. Only one of the six 10,5-day jaw grafts survived and developed a tooth. The low survival rate of the 10,5-day jaws may have been due to the

loss of their structural integrity during the three-day *in vitro* culture period, similarly noted by Brinkley *et al.* (1975), Sakakura (1986), Whitby (1987) and Peterka *et al*. (1991).

All of the *ES+JE* RS grafts that survived contained large quantities of muscle tissue. Verhoef's iron haematoxylin stain confirmed that three of these grafts consisted manly of skeletal muscle fibres, due to the appearance of striations and lack of intercalated discs. In comparison to the *ES+JE* RS grafts, only a few isolated muscle fibres were observed in the control jaw RS grafts, which originated from the mesenchyme of the jaws. It is unlikely that the presence of small amounts of myoblasts, which may have initially been included with the oral epithelium prior to *in vitro* culturing, may have proliferated to form large masses of embryonic skeletal muscle fibres in three of the *ES+JE* RS grafts. Other factors could also have resulted in the formation of the skeletal muscle fibres in the *ES+JE* RS grafts. Darabi *et al.* (2008) found that the myogenic factor *Pax3* is highly expressed in 10,5-day mouse embryos, but not in differentiating ES cells. Myogenesis in the differentiating ES cells was achieved when induced with Pax3. It has also been shown that mouse ES cells can differentiate to form normal skeletal muscle when combined with myoblasts and skeletal muscle cells and injected intra-muscularly into irradiated mice (Bhagavati and Xu, 2005). The authors also found that cell-cell contact played an important role in the differentiation of the ES cells. It is therefore possible that the inclusion of a few myoblasts could have induced the differentiation of the ES clusters, resulting in the formation of many skeletal muscle fibres.

Three of the *ES+JE* RS grafts had varying amounts of smooth muscle cells. However, even with the possible inclusion of mesenchymal tissue with the *ES+JE* grafts, the presence of large bands of smooth muscle cells in these three grafts cannot be

explained, as very little smooth muscle is found in the lower jaw mesenchyme during the embryonic stages that were used in this study (Gronthos *et al*., 2000). None of the *ES+JM* or the lower jaw RS grafts contained smooth muscle cells in such noticeable quantities. ES cells have been found to differentiate into smooth muscle cells when induced with retinoic acid (Drab *et al*., 1997), or even without external induction (Doetschman *et al*., 1985; Sinha *et al*., 2006; Xiao *et al*., 2009). It is therefore possible that the ES cells present in the *ES+JE* grafts differentiated to give rise to the large number of muscle cells observed. The presence of mucinous ciliated columnar epithelium, large quantities of smooth muscle cells and the yellow-brown gelatinous substance may also be an indication of pathological tissue (Fukushima and Fukayama, 2007; Armah *et al.,* 2009; Krishnamurthy and Saba, 2009; Liu *et al.,* 2009).

In contrast to previous studies involving the use of combinations of predetermined dental mesenchymal and epithelial cells (Yamamoto *et al*., 2003; Cai *et al*., 2007; Nakao *et al*., 2007), the present study as well as the study by Ohazama *et al.* (2004) made use of only one predetermined tissue layer in combination with undifferentiated cells. Ohazama *et al.* (2004) showed that combinations of embryonic oral epithelium with bone marrow-derived cells were able to form teeth when transplanted under the renal capsules of adult nude mice, whereas the present study showed that teeth were formed when ES cells were combined with jaw ectomesenchyme. Both the present study and Ohazama *et al.* (2004) showed that when embryonic oral epithelium was combined with ES cells, bone and soft tissue formed, but no intact tooth structures could be identified. However, when Ohazama *et al.,* (2004) used an odontoblast marker *Dspp* to label the grafts, the gene was expressed at a few sites. The authors deduced from this result that odontoblastic cell differentiation had occurred, even though no teeth had been formed.

4.2 The chorio-allantoic membrane (CAM) grafts

In comparison to the RS grafting, the CAM grafting was not as successful. Only 36% of all the CAM grafts survived, which is similar to numbers found in previous studies (Glasstone, 1954). Various factors could have contributed to the low survival of the CAM grafts. These include the ability of host blood to infiltrate the grafts (Glasstone, 1954; Peterka *et al*., 1991), the experimental techniques used, the quality of the grafts prior to CAM-grafting (Slavkin and Bavetta, 1968b), the fluctuation in temperature and the possible introduction of infection prior to or during the grafting procedure (Miller, 1969; Borges *et al.,* 2003). Large amounts of fluid introduced with the grafts may result in the grafts shifting out of position, although great care was taken in this study to introduce as little fluid to the chamber as possible. The movement of the host chick inside the egg may also have caused grafts to shift from their original position (Miller, 1969). In addition, the majority of studies that have made use of CAM grafting, used whole teeth or parts of tooth structures of embryos of more advanced developmental ages (16-21 days) (von Studitsky, 1939; Glasstone, 1954; Slavkin and Bavetta, 1968a; Slavkin and Bavetta, 1968b), compared to the embryos used in the present study (10,5 and 12,5 days). One study did however show that recombined 10-day embryonic molar ectoderm and incisor mesoderm formed molar-like structures when CAM-cultured for seven to ten days. It could however not be proven that these were developing teeth as opposed to epithelial masses, as was observed in the present study.

4.3 The three-day *in vitro*-cultured explants

The present study aimed to determine whether ES cells were able to contribute to tooth formation when combined with 12,5-day embryonic mouse lower jaw ectomesenchyme. ES cells were also combined with 10,5-day embryonic mouse lower jaw epithelium in a similar way to the study done by Ohazama *et al*. (2004), in order to compare the inductive effects that lower jaw epithelium and ectomesenchyme respectively have on ES cells.

The results obtained for *ES+JE* groups in the present study that were cultured for three days *in vitro* are morphologically similar to those observed by Ohazama *et al*. (2004). In both studies, the lower jaw epithelium could be seen in the superficial layers of the explants, while the bulk of the explants consisted of ES cells. Ohazama *et al*. (2004) was also able to show the formation of tooth buds, which was indicated through the co-expression of three odontogenic markers in the tissue, namely *Pax9, Msx1* and *Lhx7*. However, in contrast, the present study could not show any tooth development using the odontogenic marker *Msx1*, due to technical problems.

Many of the *ES+JE* and *ES+FE* and a few of the *ES+JM* and *ES+FM* three-day *in vitro*-cultured explants in the present study contained PAS-positive, amylase resistant fibers, which appeared to resemble basement membrane, present at the basal surface of some superficial and deep epithelium. Since the basement membranes would have been digested during enzymatic separation of the epithelium and mesenchyme, it is likely that the basement membranes observed in the *ES+JE* and *ES+FE* explants, could have been restored by the epithelium during the three-day *in vitro* culture period (Osman and Ruch, 1980).

Although every effort was made to remove all the epithelium from the mesenchyme of the *ES+JM* and *ES+FM* explants, it is probable that small amounts of epithelium had been included during the initial separation of the tissue layers. It would explain the presence of hair and adjacent sebaceous glands observed in two of the *ES+JM* CAM grafts, three of the *ES+JM* RS grafts and one STO+JM RS graft. Hair development is initiated in the mesenchyme (Kollar, 1970; Kratochwil *et al*., 1996; Kishimoto *et al*., 1999), unlike tooth formation, which is believed to be initiated in the epithelium (Mina and Kollar, 1987; Lumsden, 1988). This is in concurrence with the present study in which grafts that contained mesenchyme combined with ES cells showed hair development, while grafts containing epithelium combined with ES cells showed no hair. It is possible, although less likely that the ES cells could have differentiated to form epithelium, which responded to different signals from the mesenchyme in order to form hair follicles and sebaceous glands.

4.4 The number of ES cells used in the co-culture system

The ES clusters used in the current study consisted of 5-6 x 10^3 cells/cluster. Grouping of three to five clusters yielded between 1,5 x 10^4 and 3 x 10^4 cells per co-culture. In addition, the size and number of ES cell clusters used in combination with each piece of embryonic tissue was also dependent on the size of the tissue that had to be replaced by the ES cell clusters. When epithelium of the 10,5-day embryonic lower jaw or forelimb was combined with ES cells, a number of ES cell clusters were used that reflected the size of the ectomesenchyme or forelimb mesenchyme that was removed. Similarly, a comparative number of ES cell clusters were used in order to cover the whole area of 12,5-day embryonic lower jaw ectomesenchyme or forelimb mesenchyme, where the epithelium had been removed.

The number of ES cells used for each co-culture in the present study differed from that used by Ohazama *et al.* (2004). Some authors have used cell numbers from 5-6 x 10^6 cells/pellet (Pereira *et al*., 1998; Gronthos *et al.,* 2000; Ohazama *et al*., 2004) up to 5 x 10^8 cells/pellet (Nakao *et al.,* 2007). Hu *et al.* (2006) combined bone marrow stem cells with dissociated dental epithelial cells at a 1:1 ratio to make pellets of 3,8 x 10^4 cells/pellet, similar to the number of cells used in the present study (3 x 10^4 cells per co-culture). However, the present study combined only ES cells with the lower jaw ectomesenchyme without first mixing them with cells of a predetermined fate.

Ohazama *et al.* (2004) found that the combination of large pellets of aggregated ES cells (5-6 x 10^6 cells/pellet) with the epithelium of embryonic mandible primordia produced explants that were fragile and easily dissociable. Hu *et al.* (2006) found that the combination of pellets of crude bone marrow cells (5 x 10^4 cells/pellet) combined with E14 mouse dental mesenchyme also resulted in disorganisation of the pellets during *in vitro* culturing. In the present study, the use of whole ES cell clusters in combination with embryonic oral epithelium or ectomesenchyme resulted in explants that were not fragile, which also aided in the handling of these explants prior to and during transplantation under the renal capsules of adult mice. It is presumed that because the ES cell clusters used were kept whole during the combination with embryonic tissue, it allowed for the establishment of better cell-cell adhesion among the ES cells and between the ES cells and the embryonic tissue (Cui *et al*., 2004).

4.5 The identification of ES cell clusters

In the present study, groups of explants containing clusters of ES cells showed what seemed to be an eosinophilic amorphous extracellular matrix surrounding the large cells. When sections of these explants were stained with the PAS technique, the extracellular matrix stained PAS-positive and some of the cells surrounded by this extracellular matrix displayed PAS-positive granules in their cytoplasm, an indication that these were undifferentiated ES cells (Johkura *et al*., 2004; Okada *et al*., 2007). In addition, in some explants PAS-positive fibers were present in the extracellular matrix. Karbanová and Mokrý (2002) and Johkura *et al*. (2004) suggested that the differentiating ES cells may have secreted this extracellular matrix.

4.6 Oct-4 labelling of ES cells

The transcriptional regulator, Oct-4, has previously been thought to be expressed in both adult and embryonic undifferentiated cells (Pesce and Scholer, 2001; Gidekel *et al*., 2003; Pochampally *et al*., 2004; Matthai *et al*., 2006). Labelling of Oct-4 was therefore carried out to establish whether the ES cells had successfully differentiated. A lack of labelling would confirm the differentiation of the ES cells. Analysis of labelled sections revealed that there were some Oct-4 labelled nuclei and fibres present in the ES cell-containing 3-day *in vitro*-cultured explants. Positive Oct-4 labelling was also observed in sections of STO cell containing *in vitro*-cultured explants. The negative control sections that were adjacent to the Oct-4 immunolabelled sections did not show any label. In addition, the presence of Oct-4 labelled cells in the CAM and RS grafts was an unexpected observation as the ES cells were thought to have differentiated by the time these grafts were retrieved (Ohazama *et al*., 2004).

There have been discrepancies between reports on the expression of Oct-4 in various cell types (Lengner *et al*., 2007; Liedtke *et al*., 2007; Zangrossi *et al*., 2007; Lengner *et al.*, 2008; Liu, 2008; Zulli *et al*., 2008). It has been suggested that Oct-4 may not be as specific to undifferentiated cells as was thought. Some research has shown that Oct-4 is also expressed in differentiated somatic cells (Cauffman *et al*., 2006; Zangrossi *et al*., 2007), while other research has tried to disprove this (Tai *et al*., 2005; Liedtke *et al*., 2007). According to Zangrossi *et al.,* (2007), Oct-4 has a very high degree of homology with six other genes, referred to as the Oct-4 pseudogenes. The expression of these genes in differentiated somatic cells can be easily confused for Oct-4. However, when low gene expression is detected during western blot or RT-PCR assays, the Oct-4 pseudogenes should be considered (Zangrossi *et al*., 2007). It may be possible that the Oct-4 expression observed in the cells of the CAM and RS grafts could be that of one or some of its pseudogenes (Zangrossi *et al*., 2007). A detailed investigation into the Oct-4 pseudogenes and their compatibility to the Oct-4 antibody used in this study could provide some insight into this observation.

Previous studies have also suggested that the Oct-4 labelling could come from precursor cells that were originally present in the epithelial (Harada *et al.,* 2002; Jiang *et al*., 2002; Hochedlinger *et al*., 2005) and mesenchymal tissue (Jiang *et al*., 2002). The presence of Oct-4-positive nuclei in a few skeletal muscle cells of the *ES+JE* grafts, as well as in the epithelium of the collecting ducts of the adjacent host kidneys, are consistent with these studies.

Embryonic fibroblasts have the potential to dedifferentiate when exposed to ES pluripotency factors such as Oct-4. It has been shown by Neri *et al*. (2007) that if STO

cells are cultured in a medium which contains various pluripotency factors from ES cells, then the STO cells could be reprogrammed to dedifferentiate. However, the STO cells that were used as controls for the ES cells in the present study were never exposed to ES cell medium or ES cells at any point during the course of the culture period.

It has been suggested that stem cells that express Oct-4, could be targeted during the initiation of carcinogenesis (Tai *et al.*, 2005; Atlasi *et al*., 2007). Oct-4 may even be directly implicated in the formation of malignant cells (Palumbo *et al*., 2002; Kehler *et al*., 2004). In a study done by Hochedlinger *et al.* (2005), it was shown that the ectopic expression of Oct-4 in epithelial tissue prevents the differentiation of progenitor cells in the basal layers, thereby resulting the formation of dysplasia. The observation of seemingly pathological *ES+JE* RS grafts may have been linked to the expression of Oct-4 by the ES cells present.

5. CONCLUSION

The inductive effect of oral ectomesenchyme on mouse embryonic stem cells has not previously been documented. This study was thus unique in that it showed that the combination of embryonic stem cells with mouse embryonic lower jaw ectomesenchyme was able to result in the formation of complete teeth. As no teeth were observed in the negative control RS grafts, it is possible, although difficult to prove, that the ES cells may have been involved in the induction of tooth formation. In addition, the presence of Oct-4 nuclei in certain cells in the RS grafts was an unexpected observation, which could be attributed to stem cells of either host or graft origin. Future work may benefit from the use of ES cells expressing a marker such as GFP or lacZ to distinguish differentiated ES cells as a contributing factor to tooth formation when combined with embryonic lower jaw ectomesenchyme. The present findings given here may contribute to the study of tooth formation and the possible prospects of developing bioengineered teeth for replacement.

APPENDIX

A. STO-cell medium

1. Open a new bottle of 500ml Dulbecco's Modified Eagle Medium (DMEM) supplemented with 4.5g/L glucose, 0.11g/L sodium pyruvate (Ref #: 41966-029, Gibco, Invitrogen).
2. Add 500µl pen/strep to the DMEM to a concentration of 0,1% pen/strep.
3. Alliquote out 45ml units of the DMEM into plastic tubes and refrigerate until needed.
4. To make up STO-cell medium, add 5ml heat inactivated fetal bovine serum (Ref #: 10108-157, Gibco, Invitrogen) to a tube of DMEM and shake well.
5. Warm to 37°C when needed, otherwise keep refrigerated.

B. Phosphate Buffered Saline (pH 7.4)

16g NaCl

3,5g $Na_2HPO_4.2H_2O$

0,4g KCl

0,4g KH_2PO_4

2L d.H_2O

Method

1. Weigh out dry chemicals into a glass beaker
2. Add the 2 liters of d.H_2O and stir to dissolve
3. Balance the pH with a pH meter
4. Pour into bottles and autoclave
5. Aliquot and refrigerate until needed

C. Mitomycin C

1. Inject 4ml of sterilized d.d.H_2O into a new vial containing 2mg of Mitomycin C and dissolve.
2. Aliquot 1ml volumes of the solution into sterile Eppendorf tubes, wrap in foil and store in freezer until needed.
3. Add a 1ml aliquot to 49ml serum-free DMEM.
4. Aliquot 2ml volumes of the DMEM and Mitomycin C solution into sterile sterilin tubes, wrap in foil and freeze until needed.

D. ES-cell medium

1. Open a new bottle of 500ml Dulbecco's Modified Eagle Medium (DMEM) supplemented with 4.5g/L glucose, 0.11g/L sodium pyruvate (Ref #: 41966-029, Gibco, Invitrogen).
2. Add 500µl pen/strep to the DMEM to a concentration of 0,1% pen/strep.
3. Alliquote out 42,5ml units of the DMEM into plastic tubes and refrigerate until needed.
4. To make up ES-cell medium, add 7,5ml heat inactivated fetal bovine serum (Ref #: 10108-157, Gibco, Invitrogen) to a tube of DMEM and shake well.
5. Add 60µl of 0.1mM β mercaptoethanol to the DMEM.
6. Add 5µl of 0,2µM leukemia inhibitory factor (LIF) to the DMEM and shake well.
7. Warm to 37°C when needed, otherwise keep refrigerated.

E. Tissue-culture medium

1. Open a new bottle of 500ml DMEM supplemented with glutamax (Ref #: 41966-029, Gibco, Invitrogen).
2. Add 500µl pen/strep to the DMEM to a concentration of 0,1% pen/strep.
3. Alliquote out 9ml units of the DMEM into plastic centrifuge tubes and refrigerate until needed.
4. To make up the tissue-culture medium, add 1ml heat inactivated fetal bovine serum (Ref #: 10108-157, Gibco, Invitrogen) to a tube of DMEM and shake well.
5. Warm to 37°C when needed, otherwise keep refrigerated.

F. Calcium and Magnesium free Tyrode's solution

Solution A

Mix: 500ml d.H_2O

2,0g NaCl

0,05g KCl

0,012g $NaH_2PO_4H_2O$

Solution B

Mix: 480ml d.H_2O

0,025g $NaHCO_3$

Solution C

1. Dissolve 1g glucose in 20ml d.H_2O and refrigerate until needed.

Method

1. Autoclave Solution A, Solution B and d.H_2O and allow to cool.
2. Top up the levels in Solution A and B with the autoclaved d.H_2O.

3. Add Solution B to Solution A.
4. Filter Solution C using a sterile Swinnex filter and add to the mixture.
5. Add 100µL of pen/strep to the mixture once it's cooled down completely.
6. Place in a 37°C incubator 1 hour before needed.

G. Chick Ringer

Mix: 8,5g NaCl

0,42g KCl

0,25g CaCl

1L d.H_2O

Method

1. Autoclave the Ringer's solution and d.H_2O and allow to cool.
2. Top up the level in Ringer's solution with the autoclaved d.H_2O.
3. Add 100µL of pen/strep to the Ringer's solution once it's cooled down completely.
4. Place in a 37°C incubator 1 hour before needed.

H. 4% Paraformaldehyde

1. Dissolve 8g of paraformaldehyde in 200ml of PBS at 80°C under a fumehood.
2. Store at 4°C.

I. Formic citric acid

Mix: 10g Na Citrate

8ml HCl

90ml Formic Acid

110ml d.H_2O

J. Saturated Ammonium oxalate

1. Add ammonium oxalate to 100ml of d.H2O while stirring, until the solution becomes completely saturated
2. Store in an airtight glass bottle.

K. Silane-coated slides

1. Place glass slides in a glass staining rack and wash with Sunlight soap overnight.
2. Rinse the slides in tap water to remove all the soap.
3. Dry the slides in a 60˚C oven.
4. Add 2ml of 3-Aminopropyl-triethoxysilane (silane) to 100ml of acetone to make up a 2% solution.
5. Dip the glass slides in the silane solution for 30 minutes.
6. Rinse the slides in two washes of acetone.
7. Wash the slides in water.
8. Dry the slides in a 60˚C oven overnight.

L. 1% Periodic acid

1. Dissolve 1g periodic acid in a 100ml distilled water.
2. Keep the solution refrigerated until needed.

M. Shiff's Reagent

1. Add 15ml HCl to 1000ml of distilled water to make a 0,15N HCl solution.
2. Dissolve 10g of basic fuchsin in the HCl solution.
3. Dissolve 19g of sodium metabisulphate in the HCl solution.
4. Add 25g or more of activated charcoal to the solution.

5. Stir the solution overnight using a magnetic stirrer.
6. Filter the solution to remove the activated charcoal.
7. If the solution is not clear, add more activated charcoal and repeat steps 5 and 6.
8. If the solution turns pink, add a few drops of HCl.
9. The Shiff's Reagent should be placed in a bottle with a tight seal and kept refrigerated until needed.

N. 10mM Sodium Citrate Buffer

1. Add 2,94g Na Citrate to 1L of d.H_2O
2. Adjust the pH to 6.0

O. DAB

1. Weigh out 0,001g diaminobenzidine (DAB) into a clean plastic tube.
2. Before use, add 6,7µl of hydrogen peroxide (H_2O_2) to 10ml of cold d.d.H_2O.
3. Add 1ml PBS to the DAB and shake to dissolve.
4. Add 1ml of the H_2O_2 solution to the DAB solution.

P. Proteinase K

1. Dissolve 0,005g proteinase K in 100µl MilliQ water.
2. Aliquot out 10µl units into Eppendorf tubes and freeze.
3. Add 1ml PBS just before use.

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