Neoplastic tissue transfiguration *in vivo* by recombinant human transforming growth factor-β_3

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ABSTRACT

Keywords

Human transforming growth factor-β_3, human squamous cell carcinoma, tissue transfiguration, de-differentiation, neoplastic transformation.

Human oral squamous cell carcinomas (hSCCs) are the most common head and neck cancers now presenting with more aggressive biological and clinical features due to smoking and alcohol together with widespread viremia. Transforming growth factor-β (TGF-β) proteins are powerful morphogens that induce rapid and substantial induction of endochondral bone formation but in primates only.

Intramuscular heterotopic Implantation of 125 µg hTGF-β_3 generate organoids that show tissue transfiguration *in vivo* with rapid and substantial induction of mineralised bone by days 15 and 30 with large osteoid seams populated by contiguous osteoblasts, with rapid replacement and transfiguration of the *rectus abdominis* muscle into bone. Biopsies from hSCCs were implanted subcutaneously into athymic nu/nu scid mice. Rapidly growing masses were injected with 250 µg hTGF-β_3 reconstituted with 300 µl Matrigel®Matrix kept fluid on ice. Stained sections showed poorly differentiated up to anaplastic hSCCs at the periphery of the transplanted masses with a more differentiated keratinised oncotype in the centre of the growing carcinomas. qRT-PCR showed significantly up-regulation of *Keratin 17* with down-regulation of the *Peptidase Inhibitor 3* gene. The results indicate that the transfiguration patterns seen in the centre of hTGF-β_3 -Matrigel®Matrix injected specimens activates the cellular memory of the transplanted carcinomas with the induction of differentiated oncotypes with keratinised pearls of tumour growth markedly contrasting with the peripheral anaplastic carcinomatous landscape.

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Significance

Carcinomas survive by recapitulating mechanisms of normal development. The transfiguration mechanism(s) by hTGF-β_3 in Matrigel®Matrix set into motion gene expression pathways reintroducing a memory of developmental events already known to the altered cells bringing neoplastic cells back to their initial stage with keratinised pearls of a highly differentiated oncotype. The injections of 250 µg of hTGF-β_3 in Matrigel®Matrix re-introduce a memory of developmental pathways already known to the affected cells, bringing back neoplastic cells to its initial non-neoplastic and keratinised initial status.

Perspective

Malignant tumours are the leading cause of death across both developed and underdeveloped countries (https://www.cancer.gov/about-cancer/understanding/statistics). Combined chemo-, radio- and surgical treatments are not yet – if ever will be – biologically and surgically successful to therapeutically resolve human malignancies.[1]

Because of the combination of alcohol, smoking widespread viremia, and as yet unknown immunological and bacteriological causes, human oral squamous cell carcinomas (hSCCs) are now presenting with much more aggressive biological and rampant clinical features.[2] Extant features present a morphological and clinical pattern of aggressive rapid growth with anaplastic invasion. [3-5]

Experimentation in the Chacma baboon *Papio ursinus* has shown that the recombinant human transforming growth factor-β_3 (hTGF-β_3) is the most powerful osteoinductive morphogen so far tested in primates.[6,7] Our systematic studies in heterotopic *rectus abdominis* sites reported the rapid and substantial induction of bone formation with newly formed ossicles comparable to organoids. Generated organoids show tissue transfiguration *in vivo* with rapid and substantial induction of mineralised bone by days 15 and 30 with large osteoid seams populated by contiguous osteoblasts.[6,7]

EMBEDDING MOLECULAR SIGNALS INTO NEOPLASTIC MASSES: TISSUE TRANSFIGURATION *IN VIVO*

Because of the pleiotropic multifaceted biological activity of hTGF-β_3 in primates' tissues and microenvironments, experiments were set to transfigure anaplastic human oral squamous cell carcinomas (hSCCs) by direct intra-tumoral

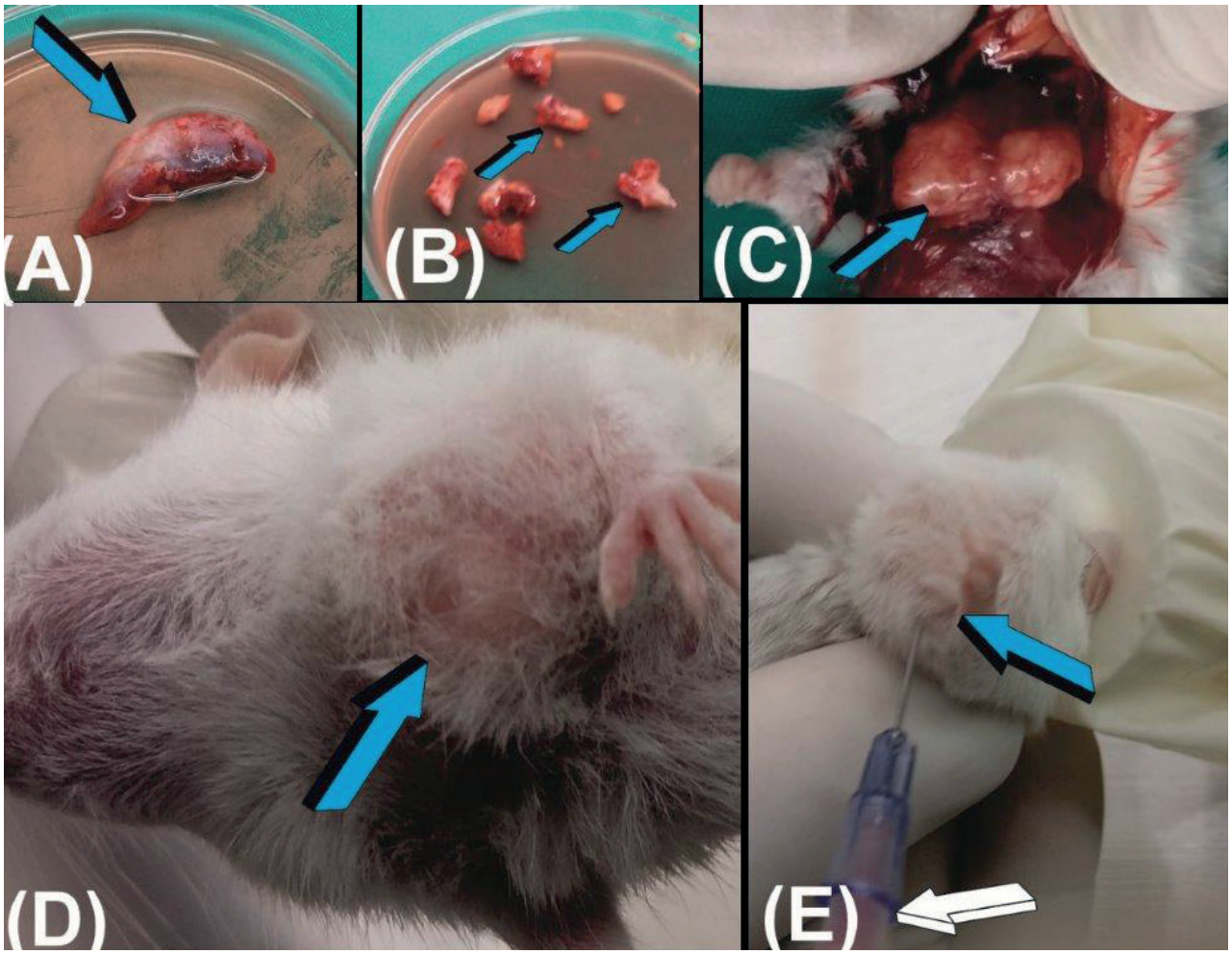

Figure 1: Transplantation of human squamous cells carcinomas (hSCCs) debrided from human patients at oncologic surgery. (A) Large fragment of a hSCC (blue arrow) brought in sterile medium to the WRAF. (B) Fragmented carcinomas (blue arrows) are transplanted subcutaneously in athymic scid nu/nu mice. (C) Bilateral growth of hSCCs over the chest of a scid nu/nu mouse 30 days after heterotopic transplantation. (D) Growing hSCC (blue arrow) after transplantation of a human SCC biopsy 30 days after transplantation just before the intra-tumoral injection of 250 µl of Matrigel®Matrix (white arrow) recombined with 250 µg recombinant human transforming growth factor-β_3 (hTGF-β_3).

injections of relatively high doses of hTGF-β_3. Human and animal ethics clearances were obtained from the University of the Witwatersrand, Johannesburg (Human Research Ethics Committee Clearance no. M150608; Animal Research Ethics Committee AREC no. 2014/39/C). Athymic *scid* mice were purchased from The Jackson Laboratories, US and kept in a sterile microenvironment at the Wits Research Animal Facility (WRAF).

Biopsies from harvested hSCCs at the time of surgical debridement (Figure 1) were implanted subcutaneously into *scid* mice over the lateral chest into the *pectoralis'* muscle opened by blunt dissection (Figure 1C). Histological analysis of transplanted hSSCs showed the classic hallmarks of highly differentiated anaplastic cells with hyperchromatic nuclei (Figures 2A,B).

Transplanted hSCCs required just over three weeks to "graft" into the host nude mice followed by growth for a further six to seven days to sizeable masses of 5/7 mm diameter (Fig. 1C). Half of the growing hSCCs in the subcutaneous space of the athymic scid mice were injected with 250 µg hTGF-β_3 reconstituted with 300 µl Matrigel®Matrix kept fluid on ice (Figures 1E). Transplanted masses were injected up to four times in selected animals. The remaining hSCCs were not injected, to monitor the carcinomatous growth of hSCCs without hTGF-β_3 injections *in vivo* (Figure 1C).

Due to a high mortality rate of the implanted mice, tissues for molecular and histological analyses were limited to two non-injected hSCCs harvested at 3 and 5 weeks after heterotopic implantation, and seven hSCCs injected and harvested at weekly intervals. Samples for molecular analysis were flash frozen in liquid nitrogen and stored at -80°C. Examination of resin-embedded sections cut at 3 to 4 µm (Morphisto AG, Germany) showed the development and growth hSCCs across the cut sections (Figures 2A,B).

The heterotopic subcutaneous growth of hSCCs is a fundamental result that shows the transplantation of viable hSCCs from bioptic surgical material (Figures 1A,B; 2A,B). Histological examination of the resin-embedded sections showed a reproducible recurrent histological pattern of undifferentiated anaplastic growth at the periphery of the transplanted hSCCs biopsies with a different yet reproducible pattern of a differentiated oncotype in the centre (Figures C,E; Figs. D,F). The morphological data showed reproducible patterns of growth spatio/temporally distributed, i.e. poorly differentiated up to anaplastic hSCCs at the periphery of the transplanted tumours (Figures D,F) with a more differentiated keratinised oncotype in the centre of the injected growing carcinomas (Figures 2C,E).

The oncotype pattern' variations are of great significance. The morphological data show reproducible patterns of growth

spatio/temporally distributed, i.e. poorly differentiated anaplastic hSCCs at the periphery of the transplanted biopsies *vs.* more differentiated with keratinised oncotype in the centre of the injected growing carcinomas, thus less malignant with a more differentiated oncotype in the centre following injections of doses of hTGF-β_3 in Matrigel®Matrix. Injected hSCCs thus induced an oncotype characterised by a shift into highly differentiated oncotypes with multiple pearls of keratinisation (Figures 2C,E). Molecular analyses were later performed on the flash frozen harvested tissues sampled according to origin. The shift into a different oncotype characterised by multiple pearls of keratinisation is mechanistically highlighted by overexpression of the human *Keratin 17* gene in hTGF-β_3 injected samples when compared to untreated hSCCs control (Figure 3).

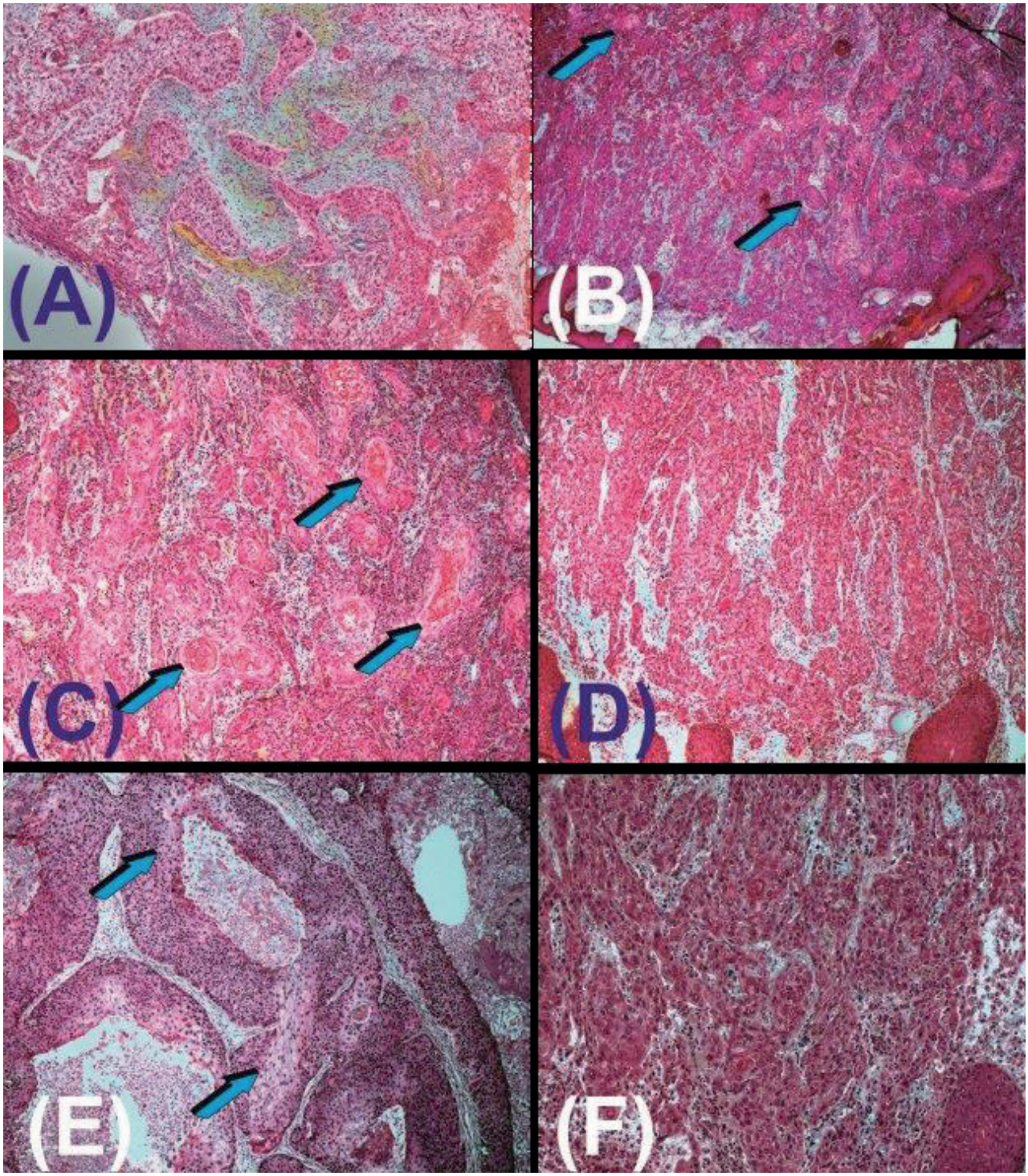

Figure 2: Composite iconographic plate showing tissue transfiguration of anaplastic human squamous cell carcinomas (hSCCs) into more differentiated oncotype after hTGF-β_3 injection in the centre of the transplanted hSCC growth. (A,B) Transplanted human squamous cell carcinomas (hSCCs). The neoplastic growths have not been injected. Image in (B) shows occasional keratin pearl formation (blue arrows). (C) Transplanted hSCC representing a section of the centre of the lesion that has been injected. The lesion is histologically well differentiated and shows increased keratin pearl formation (blue arrows) when compared to non-injected tumours. (D) Transplanted hSCC representing a section of the periphery of the neoplastic growth. The periphery of the lesion appears less differentiated than the centre of the same lesion (C). (E) Transplanted hSCC with hTGF-β_3 in Matrogel®Matrix injected into the centre of the lesion showing abundant keratin differentiation (blue arrows). (F) Periphery of a hSCC injected with hTGF-β_3 in Matrogel®Matrix showing poorly differentiated hSCC with several anaplastic cells.

RNA was extracted using the RNeasy Micro Kit (Qiagen, GmbH, Hilden, Germany). RNA quantification, cDNA synthesis and quantitative real time polymerase chain reaction (qPCR) were as performed as previously described.[8] Expression levels of *Keratin 17* and *Peptidase Inhibitor 3* normalised using three reference genes, were compared between hTGF-β_3 treated and untreated samples harvested from the scid mice and sections of the original hSCC biopsies used for the implantation. *Peptidase Inhibitor 3* was significantly down-regulated and *Keratin 17* expression significantly elevated in hTGF-β_3 treated samples compared to the untreated controls ($p < 0.01$ and $p < 0.05$, respectively) (Figure 3). The above tested genes were genes of interest identified in a genome wide expression profiling of oral squamous cell carcinoma.[9]

Non-injected hSCCs specimens showed a reproducible pattern of anaplastic growth throughout the transplanted hSCCs in the subcutaneous tissues of the operated athymic mice (Figure 2). hTGF-β_3 injected specimens showed a reproducible pattern of neoplastic growth with anaplastic differentiation at the periphery of the transplanted and injected SCCs. In the centre of the injected lesions, there was the differentiation of a highly differentiated oncotype with keratinised pearls of tumour growth markedly contrasting with the peripheral anaplastic carcinomatous landscape (Figures 2C,E). Cancers survive by recapitulating mechanisms of normal development.[10] The transfiguration mechanism(s) by hTGF-β_3 in Matrigel®Matrix set into motion gene expression pathways reintroducing a memory of developmental events already known to the altered cells bringing neoplastic cells back to their initial stage with keratinised pearls of a highly differentiated oncotype.

Endogenous TGF-β suppresses tumorigenesis in a breast cancer xenograft model by affecting cancer stem cells or early progenitors.[11] The paper reported that endogenous TGF-β has the potential to function as a tumour suppressor in carcinomas by *"depleting the putative cancer stem cells or early progenitors cell population and by promoting differentiation of the more committed progeny"*.[11] The findings that endogenous TGF-β is promoting differentiation of the more committed progeny, is also shown morphologically and molecularly in our study embedding hTGF-β_3 in fluid Matrigel®Matrix on ice resulting in the induction of differentiated oncotypes.

The injections of the 250 µg of hTGF-β_3 in Matrigel®Matrix re-introduce a memory of developmental pathways already known to the affected cells, bringing back neoplastic cells to its initial non-neoplastic and keratinised initial *status*.

ETHICS APPROVAL

Human and animal ethics clearances were obtained from the University of the Witwatersrand, Johannesburg (Human Research Ethics Clearance no. M150608; AREC 2014/39/C).

CONSENT FOR PUBLICATION

The authors agree with the contents of the manuscript and provide consent for publication. Availability of data and materials: Data are available upon request.

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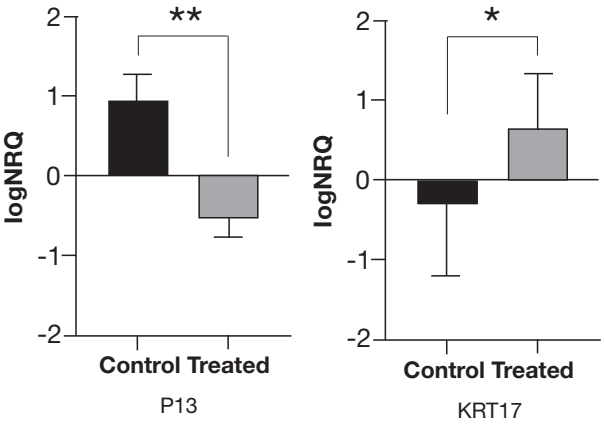

Figure 3: Comparison of *Peptidase Inhibitor 3* (*PI3*) and *Keratin 17* (*KRT17*) gene expression levels from samples harvested from hTGF-β_3-treated and untreated scid nu/nu mice and hSCC biopsy samples. The expression of *Peptidase Inhibitor 3* was significantly down regulated and Keratin 17 expression significantly elevated in hTGF-β_3 treated samples compared to the untreated controls ($p < 0.01$ and $p < 0.05$, respectively).

COMPETING INTERESTS

The authors confirm that there are no conflicts of interest.

AUTHORS' CONTRIBUTIONS

Ugo Ripamonti conceptualised, designed the study and surgically implanted the human biopsy material in scid nu/nu mice; Peter Swart analysed the histological sections; Caroline Dickens and Raquel Duarte prepared the material for molecular analyses, designed primers and performed and analysed qRT-PCR. Ugo Ripamonti wrote the manuscript and all authors commented, edited, and approved the final manuscript.

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