The use of bifunctional stem cells to produce rhino horn from endangered northern white rhinos to reduce illegal trade and the threat to rhinos living in the wild.

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ABSTRACT

The Northern white rhino has been endangered to varying degrees over the last 50 years. This subspecies is now increasingly at risk with the population dwindling. Without an end to the illegal trade in horn they are unlikely to be here for much longer. This paper suggests an alternative to this and a way to use the popularity of rhino horn and for the horn itself to help protect rhinos from poaching. By producing horn in laboratories on a commercial scale, the black market of rhino horn could be reduced if not altogether removed.

INTRODUCTION

Stem cells are unspecialised cells capable of self-renewal through cell division for a long period of time. After a stem cell divides it has the potential to become a specialised cell; such as a brain cell. Stem cells can proliferate over a few months to produce millions of specialised cells; those cells that remain unspecialised are known as parent stem cells and are capable of self-renewal. Parent cells can give rise to heart cells, nerve cells and muscle cells. Unspecialised stem cells can differentiate into specialised cells; this is controlled by the cells genes. If scientists could control the differentiation of stem cells in a laboratory they could grow cells and tissues that could have a variety of uses in medicine, including drug screening.*(Euro Stem Cell)* Embryonic stem cells (derived from embryos) are grown in the lab by transferring them into a culture medium containing nutrients for them to grow. Originally mouse embryonic skin (treated so the cells wouldn't divide) was placed in the middle of a dish and acted as a feeder layer that provided nutrients. These cells would then attach to the mouse cells at the bottom of the dish. Mouse feeder cells are no longer needed to grow stem cells. Stem cells are still produced in culture dishes unfortunately the method is inefficient as it is hard to generate an embryonic stem cell line (in which stem cells divide and multiply and are then moved to new culture dishes as the dish gets full).*(NIH)*

When an embryo is formed it is a bundle of totipotent stem cells that are able to produce more totipotent cells or differentiate into a blastocyst (an embryo that is a few days old, containing approximately 100 cells). The inner cells consist of 10-20 pluripotent cells gathered together inside the trohectoderm that will form the placenta *(Euro Stem Cell).* Pluripotent cells can self-renew to form more multipotent cells or progenitors. Pluripotent cells can differentiate into any type of cell and the stem cells produced are also known as embryonic stem cells. They are capable of producing multi potent cells that can differentiate into certain types of cells including muscle cells, nerve cells and blood cells. Multipotent cells cannot self-renew to produce more multipotent cells themselves. Pluripotent cells can also differentiate into unipotent stem cells which can only produce one type of cell.

Researchers have recently discovered bifunctional ectodermal stem cells, describing them as: 'an unreported population of quiescent cells with the basal layer of the nail proximal folds' *Leung.Y (2014).* Ectodermal stem cells have the ability to change their function by altering their homeostatic balance from nail structure to peri-nail epidermis; allowing them to perform one function more than the others *Leung.Y (2014).* Researchers were also able to isolate a protein called 'bone morphogenic protein'. That signals 'favour nail differentiation over epidermal fate'. This means that the bifunctional stem cells that are produced lean towards peri nail epidermis allowing new nail growth *Leung.Y (2014)*. This would mean that the basal layer stem cells would be able to grow into new nails. This discovery begs the question, are there more bifunctional stem cells out there?

Rhinoceros horns are able to grow back after being damaged or 'de-horned'. This is because rhino horns are primarily composed of keratin (like nails). This means that it might be possible to find bifunctional stem cells in the 'generative layer of epidermis' at the base of the horn which would aid regeneration after damage or de horning *Hieronymus.T (2006).* The bifunctional stem cells at the 'generative layer of epidermis' would cover the frontal and nasal bones of the rhino. Which is an 'interface between an ectodermal organ and the skin' where Leung suggests bifunctional stem cells are likely to be found *Leung.Y (2014).* The potential to produce rhinoceros horn in a laboratory setting opens up the possibility of sustainably managing and producing it which could influence the debate about legalising international trade of rhino horn. Poaching rhinoceros horn to sell on the black market for medicinal and traditional uses would be discouraged if manmade horn could be sold at a lower price than it is currently being sold for. This would mean that the risks of poaching horn to sell illegally would out weigh the profits and discourage poachers as a consequence. Therefore, the rhinos living in the wild would be less at a risk from poaching.

DISCUSSION

The structure of the white rhinoceros (*Ceratotherium simum*) horn had been of little importance until recently. Rhinoceros horn was thought to be made of modified hair cells. Now however there has been a lot more detailed research into the composition of Rhino horn. Rhino horn has been shown to be predominately made of keratin much like our own nails. However the horn has deposits of melanin and calcium mainly focused in its centre which gives the core of the horn slightly different properties to nail *Hieronymus.T (2006).* Melanin is a photo absorbent molecule that reduces the effects of the breakdown of the core horn caused by UV light. Calcium is a radio dense material; therefore it strengthens the core of the horn. These molecules don't only exist in the core of the horn, although that is where they are most prevalent, they can also be found throughout the rest of the horn *Hieronymus.T (2006).*

The structure of the horn also introduces another interesting discovery. Calcium and melanin are found in the amorphous keratinised inter tubular matrix between the completely keratinised tubules *Hieronymus.T (2006).* This itself creates a strong structure that when looked at more closely is made up of laminae, which have more melinised layers sandwiching a less melinised layer of inter tubular matrix. This pattern is repeated in what is thought to be a yearly growth pattern *Hieronymus.T (2006).* This creates a difficult structure to clone as differential wear is unique to each horn. Yearly growth also varies according to age, diet and where the rhino is from *Rachlow. J (1996).* This would mean that the horn produced in a lab would not exactly match the existing horn currently sold by the black market. Due to difficulty of controlling all these extraneous variables.

To be able to make rhino horn from bifunctional stem cells you must be able to create keratinocytes. In order to do this you would have to alter the homeostatic balance to obtain bifunctional stem cells that would increase rates of ectodermal growth. This could be done by reducing the signalling of a bone morphogenetic protein (BMP) *Leung. Y (2014)*. BMP's can be controlled "outside of the cell by inhibitors such as chordin" *Troilo. H (2014).* By increasing the amount of chordin in bifunctional stem cells would increase the number of inhibitors that block the cells receptors and limit cell signalling, allowing homeostatic change to occur therefore tilting the balance so horn growth is increased *Trolio. H (2014).*

Alternative ways to produce rhino horn without bifunctional stem cells are currently being investigated. This method involves the use of induced pluripotent stem cells (iPSC'S). Somatic cells converted back into their pluripotent states are able to differentiate into different types of cells, including keratinocytes, as mentioned earlier in this paper *Friedrich. I (2011).* This process has been trailed before. The human cells and the keratinocytes produced expressed markers similar to keratin 14 and keratin 5 formed in the basal layer *Kogut. I (2013)*. This shows that cells similar to bifunctional stem cells have been produced before. These cells were then encouraged to differentiate into an ectodermal fate by being treated with retinoic acid and BMP4 (a morphogenic bone protein) *Kogut. I (2013).* To convert bifunctional stem cells into keratinocytes we could also follow this process. Growing keratinocytes on collagen coated dishes is supposed to improve efficiency of differentiation as it "mimics the environment of the basal layer" *Kogut. I (2013)* In our case with rhino horn stem cells, it may provide conditions similar to the dermis covering the frontal and nasal bones of a rhino in which bifunctional stem cells are likely to be found.

The use of bifunctional stem cells dismisses the need for iPSC's as bifunctional stem cells already have the capability to differentiate into keratinocytes. This would save money and time. The money could be used to invest in the process enabling more horn to be produced. Using bifunctional stem cells to produce the rhino horn would also be more efficient than using iPSC's, as bifunctional stem cells are self-renewing a collection of them could be maintained for future use.

Once the keratinocytes have been altered to reach an ectodermal fate to promote growth of dermis, you need to sustain the growth. An epidermal keratinocyte medium is used CnT-07 as a growth medium for keratinocytes (derived from human induced pluripotent stem cells)*Kogut. I (2013)* It is likely that this would be a suitable growth medium for the keratinocytes derived from bifunctional stem cells because the keratinocytes produced from iSPC's are similar.

Once we have sustained the growth of the stem cells they then need to be modified to produce similar patterns of melanin and calcium which are distributed throughout the horn. The distribution of these molecules has been shown to be affected by local differences caused by diet, age and the amount of sunlight that the horns are exposed to *Rachlow. J (1996).* For successful production of these we would need to expose the growing horn to the same amount of UV light as a rhino would be exposed to in northern Africa. This would take into account the differences in the amount of sunlight in each season to allow the laminae in the horn to form, as it would be in its natural environment. Supplements of melanin and calcium would also have to be added. The amount of each supplement given would have to match the average amount of each molecule found in horn samples from stockpiles that the government has collected over the years *Rachlow.J (1996)*. This is because not all the melanin and calcium that are in the rhino's diet would be used for horn growth. By controlling these variables the horn grown would have similar properties to natural rhino horn.

If rhino horn was grown in a laboratory then it would grow into a cylindrical tube, as it would not be subjected to the effects of UV light and wear that give the rhino horn its distinctive shape *Hieronymus.T (2006).* Exposure to UV light causes the weakening of the outer layers of the horn which makes them more susceptible to wear, caused by typical rhino behaviour, such as foraging for food and fighting. To replicate these effects in the lab we would have to control the amount of UV light and wear down the horn with a hard material to achieve its distinctive shape *Hieronymus.T (2006).*

Rhino horn can weigh between 1 to 3 kg's. Due to this weight a scaffold could be used to support the horn as it grows *O'Connor (2014).* However the scaffold would have to be strategically placed so that it wouldn't obstruct the amount of UV light getting to the horn or prevent access to the horn. The scaffold could also be engineered in such a way that it will promote growth of the horn into the correct shape *O'Connor (2014).*

This would be a sustainable process as the bifunctional stem cells once collected, will self renew, allowing the horn to be made in batches, to produce a constant supply of horn. Horn that could then be sold to consumers at a high enough price to cover the cost of production and create a profit. The profits could to go to conservation organisations to help protect the remaining wild rhino's from poaching.

Why would people pay for manmade horn? The international ban on the trade of rhino horn was established in 1977 by CITES *Di Minin. E (2014)* but didn't put an end to the persistent demand for the horn. Rhino horn is hard to obtain therefore the cost of it has increased dramatically to extortionate rates. The manmade horn could be sold at a lower prices so that the demand for horn can be met legally, which should bring prices crashing down. Therefore the poachers will no longer have a reason to hunt rhino as it would no longer have its large price tag *Milner-Gulland. E.J (1998).*

Why is there such a demand for rhino horn? Rhinoceros horn has traditionally been thought of as having magical medicinal qualities for over 1000 years, more recently rhino horn has been rumoured to cure cancer and even hangovers, and it has become increasingly popular in Vietnam for high society parties. The popularity of rhino horn in places like Vietnam has been rising since 2008, meaning that the poachers are willing to risk more as it can be worth up to $100,000/kg more than cocaine *Guilford. G (2013)* China is commonly thought to be a large culprit for the rise in popularity of rhino horn for medicinal uses based on folk law. This is not entirely true anymore since rhino horn trade bans have been implemented in 1977. Since then from the 1980's more influence has been put on anti-poaching. This includes military style precautions being enforced by the authorities, allowing armed poachers to be shot if they are found on national parks in Zimbabwe *Rachlow.J (1996).* This has caused a noticeable depletion in the amount of horn being exported and used in China particularly since the 1990's when powdered rhino horn was removed from the pharmacopeia of Chinese traditional medicine *Guilford. G (2013).* However the popularity of rhino horn is not going to fall easily.

Since there are so many different species of rhino endangered or on watch lists a way has to be found to protect these remaining rhinos from the threat of poaching. There has been much debate over how this is going to be implemented. Recently many conservationists have adopted the policy of dehorning hoping that if the rhinos don't have their horns then they won't be targeted by poachers. The horns have been added to government stockpiles and there has even been talk about legalising the trade of rhino horn from dehorned rhinos to sell in order to make money to help protect the rhinos that are still in danger in Africa. This however would mean that the black market would become prolific unless there was some way of identifying the legal trade of regulated horns from the poached ones. The price of dehorning one rhino ranges from $350 - $1800, depending on how dispersed the population of rhinos is in that particular area *Rachlow. J (1996).* If the trade of horn is to be profitable, legal horn needs to be sold for less than the poached horn whilst still making a profit. Therefore it would need to be priced at around $2000 *Rachlow. J (1996)* possibly bringing in profits of $717,000,000 a year *Di Minin. E (2014).* If the profits were put back into conservation of rhinos it could prevent a whole species from extinction, which is currently a high possibility as the numbers of rhinos left in the wild today is continually dwindling.

In 1960 there were approximately 2,230 white rhinos in the wild *Friedrich. I (2011).* The species of white rhino is now near threatened with the northern white rhino subspecies thought to be extinct in the wild with only a four of the Northern white rhinos alive today (in a sanctuary in Kenya). Protected sanctuaries have been set up in Africa for Southern White Rhinos which are classed as near threatened, after being a conservation success story *WWF* . White rhinos predominately live in only four countries: South Africa, Namibia, Zimbabwe and Kenya *ICUN*. These countries have initiated staunch crackdowns on conservation; to reduce poaching. These have been very successful in increasing the number of white rhinos from an estimated 750 in captivity around the world in 2008 to an estimated 20,170 in the wild in 2010 *Save the Rhino*. White Rhinos are not the only endangered species of rhino: the Javan and Sumatran rhinos are critically endangered whilst the Vietnamese subspecies of Javan rhinos now all but extinct, exempt for a few on the small island of Java *ICUN*. Swift action needs to be taken to prevent any further poaching occurring as there is a high possibility that by 2024 rhinos will be extinct *Save the Rhino*. Changing attitudes and perceptions through education has been tried and will take too long to make a difference so we need a more radical approach than the current methods.

Currently the main method of deterring criminals from poaching rhino horn is by poisoning the horn with ectoparasiticides which are highly toxic and mixed with a dye (though the symptoms of ingesting it are not specifically known) *Save the Rhino*. Ed Hern, the owner of the lion and Rhino preserve near Johannesburg stated that 'the aim would be to kill, or make seriously ill anyone who consumes the horn.' The toxins injected into the horns do not affect the rhinos because their horns are cut off from their blood stream *Save the Rhino*. However the current use of this poison to deter poachers has many issues surrounding it. For example the moral issues of the poison potentially causing death to consumers of rhino horn around the world. There is also the issue that despite the horn being treated with poison, poachers may not be deterred from slaughtering rhinos to get it as the middlemen who they sell it to who would get ill. So poachers do not care if the product is poisoned or not; there is even the possibility that they might not even notice the alterations as rhino horns generally acquire damage in the wild. The dye also fades out after time *save the Rhino*. Another problem is that it is impossible to poison the horns of all the rhinos that exist and since rhino's horns grow 4-7 cm every year *Rachlow. J (1996)*, they would have to be infused with the poison about every four years. The rhino's welfare also has to be considered and it is a stressful procedure for the rhino *Save the Rhino*. Using bifunctional stem cells to mass produce rhino horn could potentially stop the criminals poaching rhinos as the horn would be readily available to buy legally.

An alternative method of conservation is to dehorn rhinos which involves the swift removal of rhinos horns with a chainsaw and then clipped *Save the Rhino*. This is not a permanent fix however as horns regrow quickly. Management of these schemes is difficult as all rhinos horns regrow at different rates depending on age, diet and various other factors *Rachlow. J (1996)*. The average range gives rise to a lot of variation and does not factor in the potential that it may not be a good idea to stress out rhinos that are pregnant or have young calves *Rachllow. J (1996)*. There is also the aspect of the cost of a method like this. It is more expensive to dehorn rhino populations that are more spread out as they need to be rounded up and are often spread out over vast areas *Rachlow. J (1996).*

CONCLUSION

There is still much work to be done on the clinical procedure for it to be possible, though most of the basis for this method of horn production has already been discovered. Issues with producing horn in laboratories include the expense of start up costs which would be greater as this is a new procedure with specialist equipment needed. If run effectively this method should be able to produce enough horn to meet demand and make it a commercially viable option by lowering manufacturing costs so that the price of the horn can be reduced to make it more competitive with the black market horn. The historical importance of rhino horn may make it difficult for some cultures to accept the use of manmade horn however it may be able to reduce the use of poached horn to low enough levels to make it not worth the poachers risk to get hold of it. The horn would want to be properly advertised to places that have a high usage of horn already like Vietnam where it could replace the use of poached horn more easily. This procedure could also be used as a basis to create different horns in the future including prized horns like the narwhale horn.

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